

# Physical degradation of genomic DNA of soybean flours does not impair relative quantification of its transgenic content

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**Abstract** In this paper, four different physical treatments (microwaves, heating by conduction, sonication and pressure autoclaving) were performed to degrade a pure DNA extract, and their influence on GMO quantification was studied. The aim was to check the hypothesis that processing of agrofood products results in a similar degradation rate for both the transgenic target and the specific target. Indeed we could observe that even if the used physical treatments could lead to a significant increase of  $C_t$  values for both transgenic and specific targets, the resulting  $\Delta C_t$  remained stable. So, the main conclusion of the study is that the aforementioned hypothesis seems valid and thus a physical degradation of DNA will not affect the relative quantitation of the GMO content, provided that both the specific and transgenic targets have very similar size. A second important issue of the experiments performed was that DNA is a very robust molecule as it is extremely difficult to reach a mean size below 100 bp. The study also gives evidence of the importance of using small targets.

**Keywords** GMO · Quantification · Real-time PCR · Roundup Ready soybean · Food analysis

## Introduction

Since 1997, labelling to indicate the presence of GMOs as such or in derived form in a food product is mandatory in the European Union. Meanwhile a more uniform regulation

is applicable to food and feed (GM food and feed regulation 1829/2003/CE [1]) for authorized events, a 0.9% threshold level below which labelling is not required if it is an accidental contamination. Quantitative methods are thus required for application of the regulation. These methods must enable one to work on processed products and not only on raw material.

Methods based on detection of proteins by immunoassays such as ELISA require the use of monoclonal antibodies that are able to detect denatured proteins [2]. With these tests quantification remains very difficult on processed products, as it is matrix dependent. Moreover, the detection is not event-specific but trait-specific.

Techniques based on PCR appear more appropriate. Double competitive PCR [3] can be used for quantification but this labour-intensive technique seems inapplicable in routine tests and remains approximate and semi-quantitative [4]. Currently, the best method for GMO quantification is real-time PCR [5] using hybridization probes, because the SYBR Green format is less specific. Here we have used real-time PCR with *Taqman* probes [6]. Targets can be very small (< 100 bp) and are therefore appropriate for detection in processed products.

The question arises, however, whether processing of food products has any influence on quantification. Due to the fact that quantification of GMOs by PCR is performed in a relative way by determination of the ratio between a construct (or event)-specific target and an endogenous target, it might be assumed that the degradation rate of both targets is similar and will not affect the final quantification result. The aim of this paper is to check this hypothesis without a matrix effect. Therefore, DNA was isolated from soybean flour of certified reference material and submitted to several physical degradation treatments that could lead to a loss of the considered targets in order to check if this could have any influence on the relative quantification. In this case, the experiments

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are directly performed on the extracted DNA and not on the matrix before DNA extraction, like in other studies [7–9].

## Materials and methods

### Soybean samples

Samples used were standards consisting of commercial transgenic certified reference material (CRM) developed by the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). They were made of powder of dried soybean (IRMM 410) containing 5.0, 2.0, 1.0, 0.5, 0.1 and 0% of Roundup Ready soybean expressed in percentage of mass.

### DNA isolation

DNA was extracted from reference material by the “High Pure PCR Template” kit of Roche Diagnostics (Mannheim, Germany) following the instructions of the kit and 40 mg was used for extraction. DNA was recovered in 200  $\mu$ l of elution buffer. Several extractions were realised and eluates were pooled in order to obtain an identical DNA concentration for the different experiments. Extractions gave a DNA yield of 10–20 ng/ $\mu$ l.

### Exposure to the microwaves

Eppendorf vials containing 80  $\mu$ l of DNA ( $\sim$ 1200 ng) coming from the extracted DNA pool were exposed to microwaves for 0–15 min at maximum power (800 W) in a microwave oven (Samsung, South Korea).

Exposures were done in steps by heating periods not exceeding 3 min with intermediate cooling periods to avoid melting of the tubes. Experiments were done on Roundup Ready soybean CRM at 0.5 and 2.0% GM content.

After the microwave treatment, volumes were readjusted with water in order to compensate the losses by evaporation.

### Conduction heating at 99 °C

Seven PCR vials of 0.5 ml each containing 100  $\mu$ l of DNA ( $\sim$ 1500 ng) were kept in the heating block of a thermocycler (Biometra T3, Göttingen, Germany) at a constant temperature of 99 °C. Every hour one tube was taken away from the thermocycler. Experiments were done on Roundup Ready soybean CRM at 0.5 and 2.0% GM content.

### Sonication

Sonication was performed on an initial volume of 1000  $\mu$ l ( $\sim$ 15  $\mu$ g of DNA) at maximum power (170 W) on a

Branson Sonifier 250 (Branson Ultrasonics Corporation, Danbury, CT, USA). Aliquots of 100  $\mu$ l were taken at 5, 15, 30, 60, 120, 240, 360 and 480 min.

Experiments were done on Roundup Ready soybean CRM at 0.5 and 2.0% GM content.

### Autoclaving

Aliquots of 100  $\mu$ l ( $\sim$ 1500 ng) were oven-dried at 55 °C. The tubes were then autoclaved at 120 °C during times ranging from 10 to 80 min (separate autoclavings). The autoclaved DNA samples were resuspended in 200  $\mu$ l of water. Experiments were done on Roundup Ready soybean CRM at 0.5, 2.0 and 5.0% GM content.

### Gel electrophoresis

All treated DNA were electrophoresed in 1% agarose gel (Sigma-Aldrich, Bornem, Belgium) for 90 min at 400 mA, and stained with ethidium bromide for visualisation.

### Oligonucleotides

A part of the lectin gene was used as specific (or endogenous) target for soybean detection and the 35S-CTP junction for construct-specific detection of the Roundup Ready target. The sequences of primers and probes are presented in Table 1 and their relative localisation is shown in Fig. 1. Validated primers and probes producing amplicons of  $\sim$ 80 bp are those reported in the AFNOR standard [10] concerning methods of analysis for the detection of genetically modified organisms and derived products. Primers for  $\sim$ 170 bp targets were designed by Wurz et al. [11] for the 35S-CTP junction and those for lectin fragment were designed by us with the help of the Primer Express software.

These primers were used with the same probes as the  $\sim$ 80 bp targets. Primers were synthesised by Invitrogen (Merelbeke, Belgium). Probes were purchased from Eurogentec (Liège, Belgium). They were labelled with the reporter dye FAM at the 5' end and the quencher dye TAMRA on the 3' end.

### PCR conditions

PCR reactions (35  $\mu$ l) were performed on the ABI5700 thermocycler (Applied Biosystems, Foster City, CA, USA) with the Taqman Universal PCR Master Mix (Applied Biosystems-Roche Molecular Systems, Branchburg, New Jersey, USA). At 17.5  $\mu$ l of Master Mix, we added 2.5  $\mu$ l of each primers (5 mM), 2.2  $\mu$ l probe (5 mM), 5.3  $\mu$ l PCR quality water and 5  $\mu$ l DNA (expressed on a per well basis).

**Table 1** Sequence of the primers and probes used

Type of target	Target	Primers and probe	Sequence	Amplicon size (bp)
Specific	Lectin	Lectin-F	5'-TCCACCCCCATCCACATTT-3'	81
		Lectin-R	5'-GGCATAGAAGGTGAAGTTGAAGGA-3'	
		Lectine-TMP (probe)	5'-FAM-AACCGGTAGCGTTGCCAGCTTCG-TAMRA-3'	
Transgenic	35S-CTP junction	RRS-F	5'-GCCATGTTGTTAATTTGTGCCAT-3'	83
		RRS-R	5'-GAAGTTCATTTTCATTTGGAGAGGAC-3'	
		RRS-TMP (probe)	5'-FAM-CTTGAAAGATCTGCTAGAGTCAGCTTGTTCAGCG-TAMRA-3'	
Specific	Lectin	Lec2-F	5'-AAAACCCTCGTCTCTTGGTGC -3'	173
		Lec2-R	5'-TTAGTGTCAATTGGTGCGAGAAAG-3'	
		Lectine-TMP (probe)	5'-FAM-AACCGGTAGCGTTGCCAGCTTCG-TAMRA-3'	
Transgenic	35S-CTP junction	RR2-F	5'-TGATGTGATATCTCCACTGACG-3'	171
		RR2-R	5'-TGATCCCTTGAGCCATGTTGT-3'	
		RRS-TMP (probe)	5'-FAM-CTTGAAAGATCTGCTAGAGTCAGCTTGTTCAGCG-TAMRA-3'	

Note: For a schematic representation of the position of the different primers and probes see Fig. 1.

Reactions were performed in 96-Well Reaction plates (Applied Biosystems) covered by optical caps or optical films (Applied Biosystems). Filled plates were centrifuged (500 rpm, 10 s) to eliminate possible air bubbles in the bottom of the wells.

PCR conditions were standard: 2 min at 50 °C, 10 min at 95 °C and 50 cycles of 15 s at 95 °C and 1 min at 60 °C. PCR Reactions were performed in separate wells for the endogenous and transgenic targets as the ABI 5700 is not fit for duplex reactions.

Once the run ended, the baseline (normalised fluorescent signal before occurrence of the exponential PCR amplification) was adjusted and a threshold level of fluorescence was fixed in the middle of the linear region of the logarithmic curve of amplification.

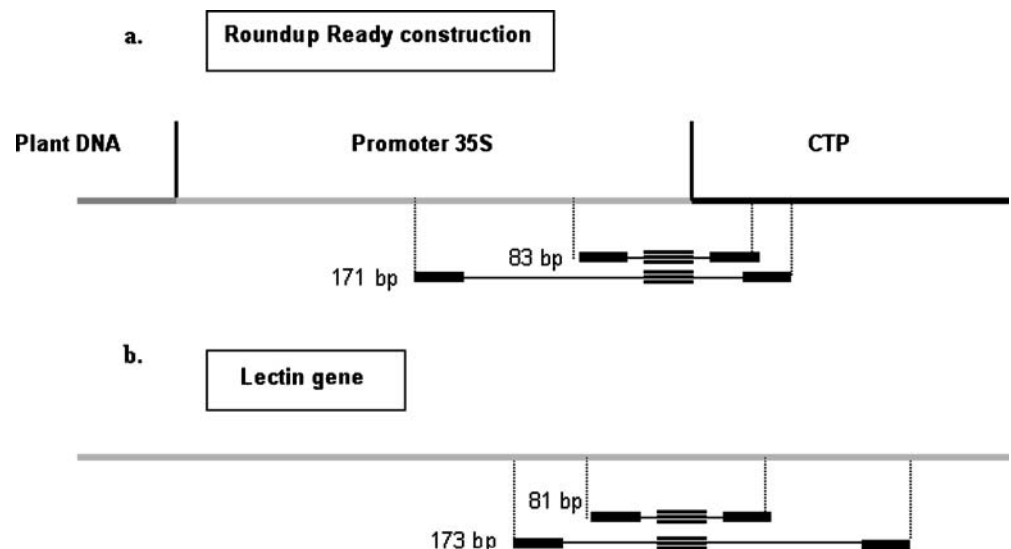
**Results and discussion**

In commonly used quantification methods, standards are based on diluted genomic DNA [12, 13], plasmid DNA [14] or hybrid amplicons [15]. Quantification is possible by comparing values obtained in the respective standard curves for transgenic and specific targets.

To quantify GMO, we used the  $\Delta C_t$  method [16]. As in the previous methods, two targets were amplified, a transgenic and a taxon-specific one. We used a segment of the lectin gene as endogenous target and the 35S-CTP junction for the construct transgenic target (Table 1, Fig. 1).

The amplification curves were used to determine a cycle threshold value ( $C_t$ ) for each target. The  $C_t$  is the cycle number required to reach a defined amount of fluorescence

**Fig. 1** Schematic representation of the ~80 and 170 bp amplified fragments with TaqMan probes format. **a** Structure of the amplified 35S-CTP junction of Roundup Ready soybean. **b** Amplified lectin gene fragments



**Table 2** Mean  $C_t$  and  $\Delta C_t$  after exposure to microwaves (2.0% RR soybean,  $n = 2$ )

Minutes of exposure	Mean $C_t$ (L)	Standard deviation	Mean $C_t$ (RR)	Standard deviation	$\Delta C_t$	Standard deviation
0	22.16	0.17	29.79	0.35	7.63	0.18
1	22.42	0.57	29.71	0.08	7.30	0.49
2	22.11	0.07	29.45	0.16	7.34	0.08
3	22.15	0.20	29.95	0.07	7.80	0.27
5	22.23	0.31	30.00	0.03	7.77	0.28
7	22.22	0.12	29.91	0.19	7.69	0.31
9	22.12	0.08	30.05	0.07	7.94	0.15
12	22.20	0.23	29.99	0.05	7.79	0.28
15	22.06	0.11	29.49	0.03	7.43	0.14

Note: L lectin target, RR construct specific target. Real-time amplification was performed with the  $\sim 80$  bp targets.

itself ranging within the exponential amplification phase. The difference of both  $C_t$ , the one of the transgenic target and the one of the specific target, gives a parameter called  $\Delta C_t$  that we correlate in a linear fashion to the logarithm of the concentration of GMO (here expressed as a percentage in mass). Quantification is possible once a calibration curve is made with extracts obtained out of reference material with certified GMO amounts (0.1, 0.5, 1.0, 2.0 and 5.0%).

As we aim to check if the physical treatments might influence relative GMO quantification, it is sufficient to see whether the treatments have any influence on the obtained  $\Delta C_t$ .

We express the postulate that both the taxon-specific and the transgenic targets undergo a similar degradation rate. This should result in a later detection of targets submitted to a longer exposure to the physical treatment, but the  $\Delta C_t$  should remain constant and thus result in an equal quantification of the GMO content whatever the exposure time is to the physical process.

Experiments were done on extracted DNA. This is more sensitive to physical processing. DNA used was extracted from certified reference flours of soybean at

different GMO contents but for the sake of presentation only results on 2.0% of Roundup Ready Soybean content are fully given.

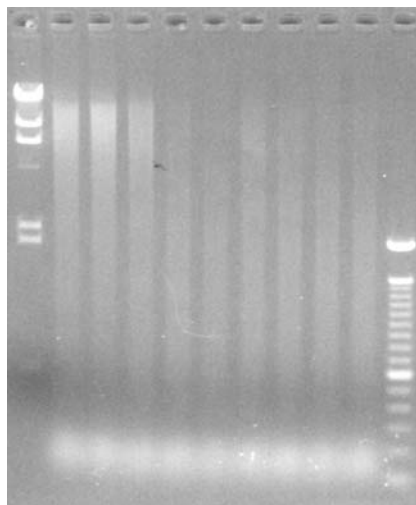
#### Exposure to the microwaves

Tubes containing DNA were exposed to microwaves for up to 15 min at maximum power as described in the “Materials and methods” section. Afterwards, this DNA was used in a real-time PCR with the  $\sim 80$  bp targets.  $\Delta C_t$  remained stable (Table 2) but exposure to the microwaves was not really a conclusive treatment. Indeed, expanding the exposure time did not result in an increase of the  $C_t$ 's of the lectin or the transgenic target.

DNA quality criterion was assessed on agarose gel to gain more information on its structural integrity. DNA treated with microwaves yielded more severely degraded material (Fig. 2) but with still a great amount of DNA segments much larger than the target size. It was however difficult to perform longer treatments because exposure time had to be limited due to resistance of the plastic tubes to heating by microwaves.

**Fig. 2** Appearance of soybean genomic DNA on agarose gel after several defined exposure periods to microwaves (2.0% RR soybean)

M1 1 2 3 4 5 6 7 8 9 M2



**M1:** MW Marker  $\lambda$  *Hind* III

**1:** 0 min microwaves

**2:** 1 min microwaves

**3:** 2 min microwaves

**4:** 3 min microwaves

**5:** 5 min microwaves

**6:** 7 min microwaves

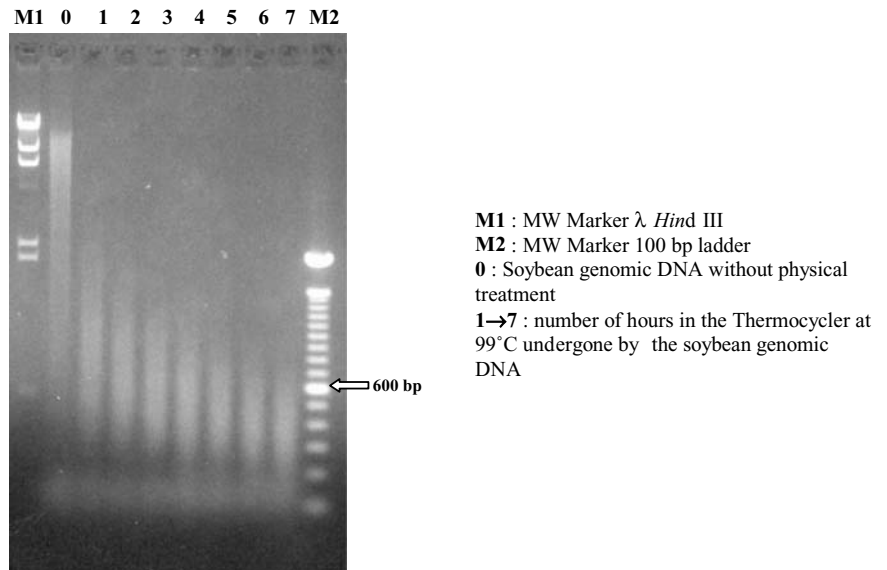
**7:** 9 min microwaves

**8:** 12 min microwaves

**9:** 15 min microwaves

**M2:** MW Marker 100 bp ladder

**Fig. 3** Migration of DNA on agarose gel after heating at 99 °C (2.0% RR soybean)



**Conduction Heating at 99 °C**

In order to increase the effect of the physical treatment on the  $C_t$  values, DNA solutions were heated continuously at 99 °C for 1–7 h on the heating block of a thermocycler as described in the “Materials and methods” section.

Putting an aliquot of each tube of DNA on agarose gel (Fig. 3), illustrates the DNA degradation. Nevertheless, even after 7 h of heating, the mean size of the DNA segments is still about 400 bp. This size is still too large to have an impact on the resulting  $C_t$ 's and thus also on the  $\Delta C_t$  when using the 80 bp targets (Table 3).

**Sonication**

Analysis of the sonicated material by agarose gel electrophoresis shows the degradation effect of sonication (Fig. 4). It can also be observed that the effectiveness of sonication decreased with time. There is no great difference between material sonicated during 4 and 8 h.

For this experiment, two target sizes were amplified by real-time PCR: one of ~80 pairs of bases (bp), identical

to those which were targeted in the previous experiments, and another one of ~170 bp (see “Materials and methods” section).

This time we can observe an increase in the  $C_t$  with the length of the physical treatment. Increasing of the  $C_t$ 's is more pronounced for the ~170 bp fragments than that for the ~80 bp fragments (Table 4 and Fig. 5). When compared to a DNA sample submitted to the same treatment, detection of the smallest target (~80 bp) is always earlier than detection of the ~170 bp amplicon. However, when compared at a given target size, the impact on the  $C_t$  of a physical treatment is similar for the specific and transgenic targets. This leads to a stable  $\Delta C_t$ .

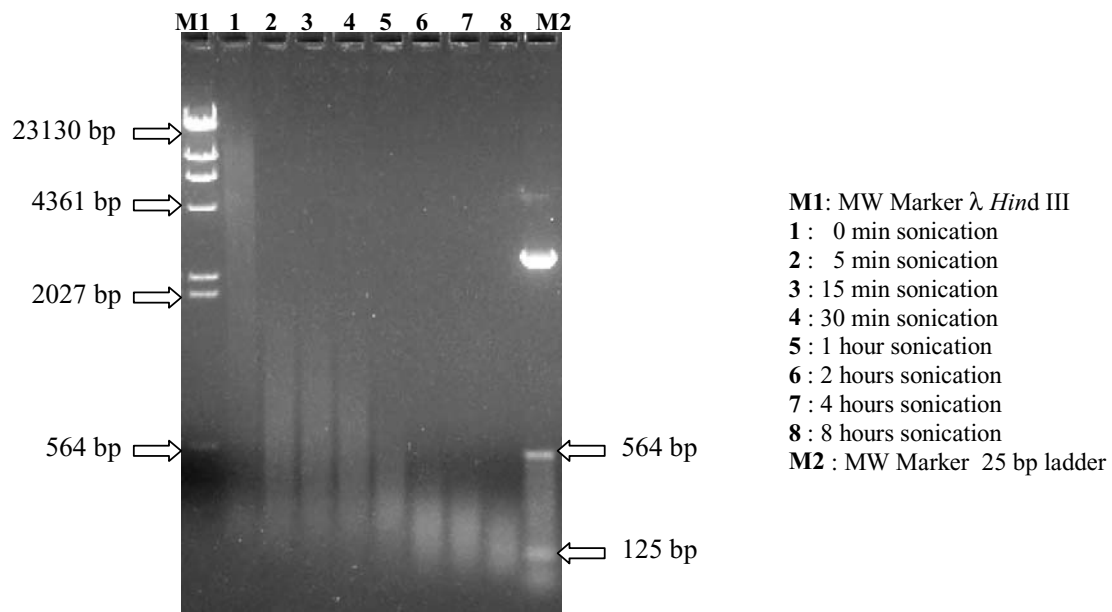
**Autoclaving**

An advantage of autoclaving is that it influences in an even more significant way the resulting  $C_t$ . The specific and transgenic targets of 80 bp (soybean 2.0% RR) underwent an increase of  $C_t$  of ~8.5 cycles after a treatment for 60 min of processing (Table 5). Autoclaving being a drastic treatment,

**Table 3** Mean  $C_t$  and  $\Delta C_t$  after heating at 99 °C on the heating block of a thermocycler (2.0% RR soybean,  $n = 2$ )

Number of hours at 99 °C in the PCR apparatus	Mean $C_t$ (L)	Standard deviation	Mean $C_t$ (RR)	Standard deviation	$\Delta C_t$	Standard deviation
0	24.17	0.03	32.35	0.23	8.18	0.20
1	24.66	0.47	31.74	0.47	7.84	0.01
2	24.54	0.03	32.46	0.28	7.92	0.25
3	24.70	0.07	32.32	0.24	7.62	0.17
4	24.52	0.01	32.66	0.32	8.14	0.31
5	24.52	0.07	32.37	0.19	7.85	0.12
6	24.40	0.02	32.26	0.49	7.87	0.47
7	24.48	0.05	32.18	0.17	7.71	0.22

L lectin target, RR construct specific target. Real-time amplification was performed with the ~80 bp targets



**Fig. 4** Migration of DNA on agarose gel after sonication (2.0% RR soybean)

**Table 4** Mean  $C_t$  and  $\Delta C_t$  after DNA sonication (2.0% RR soybean,  $n = 3$ )

Minutes of sonication	Mean $C_t$ (L)	Standard deviation	Mean $C_t$ (RR)	Standard deviation	$\Delta C_t$	Standard deviation
Targets of $\sim 80$ bp						
0	20.80	0.40	28.05	0.42	7.26	0.48
5	21.01	0.71	28.28	0.54	7.27	4.13
15	21.15	0.29	28.78	1.92	7.64	6.68
30	21.10	0.25	28.38	0.42	7.28	1.91
60	21.03	1.23	28.54	2.26	7.51	7.30
120	21.70	0.75	28.73	0.33	7.03	3.15
240	21.92	0.48	29.14	0.59	7.22	1.21
480	22.36	0.72	29.58	2.51	7.21	9.49
Targets of $\sim 170$ bp						
0	22.07	0.20	28.63	0.66	6.55	2.18
5	22.51	0.58	29.08	1.84	6.56	6.31
15	22.47	0.69	28.93	0.95	6.46	2.06
30	22.64	0.48	29.22	2.76	6.58	12.85
60	23.37	0.53	30.40	0.49	7.03	2.48
120	24.77	0.42	32.04	1.65	7.27	8.70
240	25.77	0.78	32.61	0.75	6.84	6.49
480	26.69	0.66	33.58	1.97	6.92	7.47

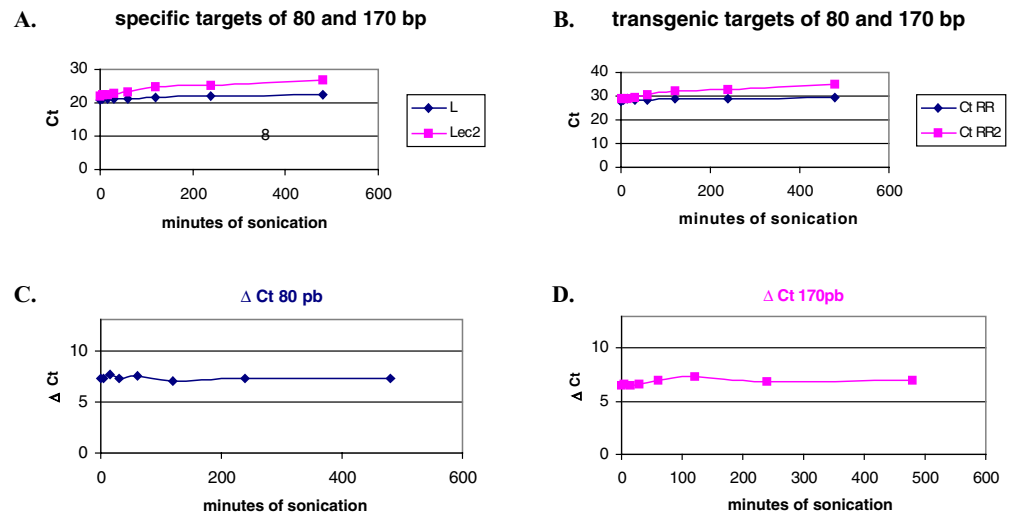
**Table 5** Mean  $C_t$  and  $\Delta C_t$  after autoclaving ( $n = 4$ )

Minutes of autoclaving <sup>a</sup>	Mean $C_t$ (L)	Standard deviation	Mean $C_t$ (RR)	Standard deviation	$\Delta C_t$	Standard deviation
80 bp						
0	21.21	0.15	28.31	0.16	7.11	0.16
20	22.64	0.27	29.86	0.32	7.22	0.52
40	25.92	0.43	33.05	0.53	7.13	0.91
60	29.97	0.31	36.83	0.41	6.85	0.37
80	37.54	0.73	Not detected			

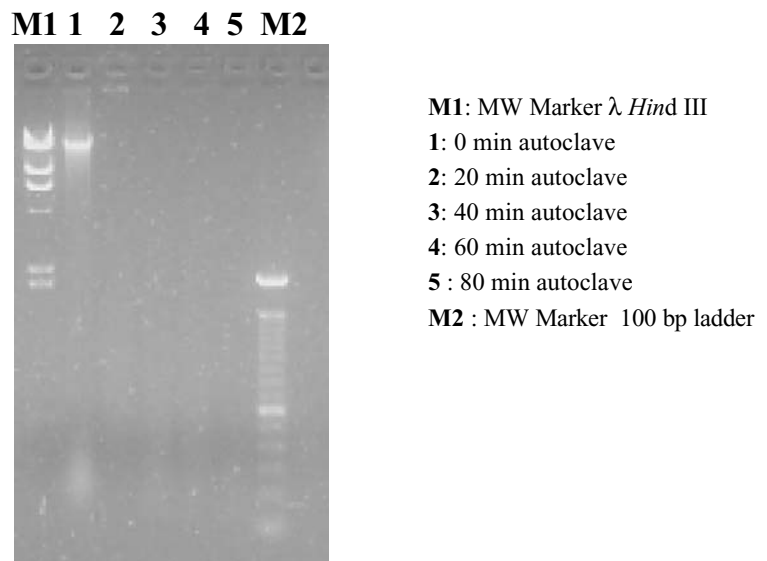
<sup>a</sup>2% RR soybean



**Fig. 5**  $C_t$  and  $\Delta C_t$  of 80 and 170 bp fragments amplified from 2% RR soybean. **A** Increase of  $C_t$  for specific targets (lectin) of 81 bp (L) and 173 bp (Lec2) with length of treatment. **B** Increase of  $C_t$  for transgenic targets (35S-CTP) of 83 bp (RR) and 171 bp (RR2) with length of treatment. **C** Evolution of  $\Delta C_t$  for targets of ~80 bp (L and RR). **D** Evolution of  $\Delta C_t$  for targets of ~170 bp (Lec2 and RR2)



**Fig. 6** Migration of DNA on agarose gel after autoclaving (2.0% RR soybean)



it also led to some non-detection for long-time processes (Table 5).

A difference of ~16 cycles was even observed for DNA of 5% RR Soybean CRM (data not shown) where transgenic target was always detectable after 80 min, probably due to the higher GMO level.

Influence of autoclaving has also been observed on agarose gel (Fig. 6) where DNA was hard to visualize after a treatment of 20 min. However, we still have a stable  $\Delta C_t$  for experiments where it was possible to determine  $C_t$ 's.

Autoclaving shows that with the use of suitable targets it is possible to carry out a quantification on products if there remains a sufficient number of intact targets after degradation. This number of suitable targets can be increased by the usage of smaller targets.

Statistical verification to check absence of influence of the treatments on  $\Delta C_t$

Statistical *t* tests were performed to verify the absence of influence of the various treatments on  $\Delta C_t$ . Slopes of the linear regression ( $y = ax + b$ ) between  $\Delta C_t$  and duration of treatment were calculated. Hypothesis of slope = 0 (no influence of treatment) was tested. Standard errors, *t* values and probabilities with *n* - 2 degrees of freedom were calculated for the various experiments with the GLM procedure of SAS v8.02 (Table 6). A probability superior to 0.05 or an observed *t* value superior to *t* value of Student table with *n* - 2 degrees of freedom indicate that hypothesis  $H_0: a = 0$  is verified. For the different treatments, the hypothesis was validated with ~80 and ~170 bp targets and no influence of treatments on  $\Delta C_t$  was highlighted for DNA from certified reference material at 0.5 and 2%.

**Table 6** Statistical tests to verify absence of effects of treatments on  $\Delta C_t$ 

RR (%)	Treatment	<i>a</i>	Standard error	( <i>n</i> - 2) df	<i>t</i> value	$t_{1-\alpha/2} = 0.975$	Probability
0.5	Microwaves	0.0185	0.013028	7	1.42	2.37	0.177
2	Microwaves	0.0108	0.013028	7	0.83	2.37	0.421
0.5	Heating at 99 °C	-0.0576	0.039957	5	-1.44	2.57	0.175
2	Heating at 99 °C	-0.0055	0.039957	5	-0.14	2.57	0.893
0.5	Sonication (~80 bp)	0.0010	0.000637	6	1.50	2.45	0.1466
2	Sonication (~80 bp)	-0.0004	0.000717	6	-0.58	2.45	0.5701
0.5	Autoclaving (~80 bp)	-0.0107	0.006659	3	-1.61	3.18	0.1521
2	Autoclaving (~80 bp)	-0.0043	0.009417	2	-0.46	4.30	0.6582

*a* slope, df degrees of freedom, hypothesis  $H_0: a = 0$  is verified if probability is superior to 0.05.

## Conclusions

All these experiments give evidence that DNA is extremely robust to physical degradation treatments. The results of this study show that at least without a matrix effect (that was not taken into account here) it may be considered that physical degradation of DNA will not result in a significant difference of the relative GMO quantification provided that there are still enough targets to perform a reproducible quantification.

However, endogenous and transgenic targets should have about the same size. Different sizes of amplicons could generate a bias in the quantification due to the fact that, on hardly processed samples, smaller amounts of the larger target will be available, whilst using targets of the same size and efficiency, it will not affect the ratio between these targets.

Amplification of small fragments (< 100 bp) is preferred because in products with degraded DNA an earlier detection is observed.

As DNA extraction itself may be a source of DNA degradation, these results support the independence of extraction and PCR modules which is a prerequisite to perform validation in a modular approach [17].

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