

# PCR detection of soy ingredients in bread

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**Abstract** Bread may contain soy ingredients to variable extends. The nature of this soy may be genetically modified, or the ingredient may cause allergic reactions in sensitive patients. PCR is a powerful tool to detect the presence of soy in food products. Major prerequisite for a PCR analysis is DNA of good quality and quantity. The persistence of soy DNA during bread making was evaluated using different amounts of different soy ingredients. Agarose gel electrophoresis of the samples taken during the baking process reveal that DNA is degraded, but subsequent PCR detection remains possible. Results, however, greatly depend on the type of ingredient and the amount present. Lower detection limits were obtained for full-fat and defatted soybean flours than those for toasted soybean flour and soy fibre samples. Samples taken at different places in the bread, however, reveal that sampling strategy may influence the PCR results.

**Keywords** GMO · Allergens · Soy · Bread · DNA degradation · PCR

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## Introduction

Soy can be used as a baking aid, serving different purposes. Soybean flour is known for its excellent foaming abilities; soy proteins help to improve and maintain moistness and softness of the product due to their water binding properties and improve crust colour and toasting characteristics in bread [1]. However, soy is recognised as one of the major allergens which can be present in food and should therefore be labelled according to the European Directives 2000/13/EC and 2003/89/EC [2, 3]. Moreover, if the origin of the soy is genetically modified, labelling is also required according to European Regulations 1829/2003 and 1830/2003 [4, 5].

The stability of DNA makes it the preferred analyte for PCR analysis. The polymerase chain reaction is therefore the method of choice for the detection of genetically modified organisms (GMOs). Furthermore, this technique is also involved in the analysis of allergens in foodstuffs. Although PCR is not able to detect the through allergenic compound in a product, it can be used to detect the species from which the allergenic compound is derived. It can therefore be used as an important tool for the screening of food products for the presence of allergens. Moreover, PCR is known to be a very sensitive method, especially compared with protein-based methods, such as the sandwich ELISA technique, mostly used for allergen detection [6, 7].

Nevertheless, damage within the DNA fragments is believed to be caused by the exposure to heat or pressure, enzymatic degradation by nucleases, ionic strength, chemical agents, water activity, shear forces and pH values [8–10]. Hydrolysis (non-enzymatic and enzymatic), oxidation and non-enzymatic methylation of DNA are examples of reactions occurring at significant rate under these conditions [11]. These reactions may affect biochemical components in a food product: proteins are

denatured and starch is gelatinised [10]. The degradation of DNA may lead to the inability to perform a PCR analysis.

In this study, different soy ingredients were added to bread: full-fat soybean flour, defatted soybean flour, toasted soy, soy protein isolate and soy fibre. They were used in bread to establish practical detection limits of the soy lectin gene, a reference gene used to evaluate the presence or absence of soy with high sensitivity [12, 13]. In order to thoroughly evaluate the influence of the baking process on the (soy) DNA in bread, samples were taken during different steps of the baking process, and also at different places in the bread. Finally, some commercial wheat flour samples containing soy were used to evaluate the persistence of the soy-DNA during the baking process.

## Materials and methods

### Bread preparation [14]

All dry ingredients (280.0 g wheat flour, 2.8 g yeast and 0.56 g malt) were first mixed during 2 min in a Chopin mixer before the liquid ingredients (4.2 g salt dissolved in 55 ml water and 0.007 g ascorbic acid dissolved in 100 ml water) were added in the next minute. Mixing was then stopped during 1 min to scrape down dough and ingredients. After this intervention, the dough was again mixed during 3 min. Temperature of the dough after mixing should be between 26.5 and 27.5 °C before putting it to rise for 10 min at 30 °C. The dough was then split into two parts, one part of 250 g and the rest part (about 175 g), both transformed into rolls with a Brabender roller and set to rise again for 30 min at 30 °C. After this second rising, both pieces of dough were stretched out longitudinally and placed in between two gauge strips of 5 mm, enabling the dough to be rolled out by pressing the dough from the middle out to the top with a rolling pin. This was done 12 times in the opposite directions of the stretched dough. The two flat pieces of dough were then rolled with the hand palm into a compact roll without air spaces in the centre. The doughs were then placed on a baking tray with the joint placed on the bottom and put in the rising cabinet for another 65 min. Doughs were then put in a pre-warmed oven and baked according to the following process: 2 min at 230 °C with steam, 13 min at 200 °C and 5 min at 230 °C. The breads were placed on a grid to cool down to room temperature.

A part of the wheat flour was exchanged to variable extents with a soy ingredient, to determine the influence of the entire baking process on these ingredients. The used amounts were, expressed as weight percentage on total dry weight, 0.2 wt%–0.4 wt%–0.6 wt%–1.0 wt%–2.0 wt%. Five commercial soybean-derived ingredients were used: full-fat (enzyme active) soybean flour, defatted soybean

flour, toasted soybean flour, soy protein isolate and soy fibre.

### Sampling

Samples were taken at different stages during the baking process and at different places in the bread, and stored in the refrigerator at 4 °C until DNA extraction within the next 24 h.

### DNA isolation

DNA was extracted from the dough and bread by a CTAB method proposed by the CEN and ISO for GMO detection [13, 15]. The concentration of DNA was checked by spectrophotometry and the integrity by loading 10 µl of the extract on a 2% agarose gel stained by ethidium bromide. For the spectrophotometrical analysis samples were diluted 1/10 or 1/20 in mQ water. The (im)purity factors (absorbance proportions  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$ ) were recorded as well.

### PCR analysis

The primers plant c (5'-CGA AAT CGG TAG ACG CTA CG-3') and plant d (5'-GGG GAT AGA GGG ACT TGA AC-3') detect a 500–600-bp fragment of a non-coding region of the chloroplast genome [16]. The primers GMO3 (5'-GCC CTC TAC TCC ACC CCC ATC C-3') and GMO4 (5'-GCC CAT CTG CAA GCC TTT TTG TG-3') are specific for the detection of the single-copy lectin gene *Le1* and yield a PCR product of 118 bp [12].

For the PCR amplifications, the following PCR mix was composed: 2 µl of extracted DNA product was added to 36.6 µl bidistilled water, 5 µl of 10 × GeneAmp<sup>®</sup> PCR buffer I (Applied Biosystems, Lennik, Belgium), 2 µl of each primer (10 µM), 2 µl dNTPs solution (5 mM) and 0.4 µl AmpliTaq<sup>®</sup> DNA polymerase (5 U/µl; Applied Biosystems). The PCR thermal cycle program for the plant c/d primers consisted of: initial denaturation at 95 °C for 3 min, 35 cycles of a denaturation at 95 °C for 20 s, primer annealing at 54 °C for 40 s and extension at 72 °C for 30 s, followed by a final extension of 3 min at 72 °C. For the GMO3/4 primers, after initial denaturation (95 °C for 3 min), 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s and extension at 72 °C for 60 s were followed by a final extension of 3 min at 72 °C.

The absolute detection limit ( $LOD_{ABS}$ ) for the GMO3/4 PCR, determined through a decimal dilution series, is 408 copies of the lectin gene [13].

DNA fragments were separated by gel electrophoresis on a 2% agarose gel with TAE buffer, stained with ethidium

bromide and visualised using the ImageMaster VDS UV transilluminator system of Amersham Pharmacia Biotech (Roosendaal, The Netherlands). DNA fragment length was checked with a 100 bp DNA ladder, marked as “M”.

## Results and discussion

### Influence of heat on DNA degradation

Heat is not equally transferred in a bread. In order to evaluate the influence of heat on DNA in bread, a dough was made with 0.2 wt% full-fat soybean flour. Samples were taken at different levels: at the top crust, the bottom crust, just under the top crust and in the middle of the bread (Fig. 1). From the different samples taken, DNA was extracted and the integrity and quality were evaluated through agarose gel electrophoresis and PCR analysis.

Results in Fig. 2a show DNA smears in lanes 1 and 2 for the extracted DNA from the top crust. Clearly, the top layer of the bread suffers the most from the heat treatment, as DNA is most degraded for these samples compared with the others. Differences in intensity between samples in duplicate are most probably due to different extraction efficiency. An increasing intensity  $I$  of the DNA smears is observed in the order:

$$I_{\text{top crust}} < I_{\text{bottom crust}} < I_{\text{middle}} < I_{\text{under crust}}$$

The highest intensity of DNA can be noted for the sample taken just under the upper crust. Although more subjected to heat, the smear for this sample is more intense than for the sample taken in the middle of the bread. Also many more DNA fragments of higher molecular weight are present.

After the test for the integrity of the isolated DNA, the quality of the samples was tested through PCR with the GMO3/4 primers, detecting the soy lectin gene. Results are depicted in Fig. 2b. Positive amplification can be obtained for samples taken just under the top crust, at the bottom

crust and in the middle of the bread. For the top crust, a positive as well as a negative signal are obtained. This can be explained by the extraction efficiency, which was higher for the *a* sample (lane 1) than for the *b* sample (lane 2), as can be deduced from the results of the agarose gel. There, the *a* sample gives a more intense smear than the *b* sample. Again, differences in intensity can be observed between the positive signals. Although for a qualitative result only an answer should be given as “present” or “not present”, it is clear that the chance of having a positive result is the highest for the samples taken just under the top crust.

The poor results for the samples taken at the top crust can be easily explained by the heat exposure at this point of the bread. The difference between bottom and top crust could be due to the difference in heat transfer: where the top crust is directly exposed to heat by convection, the bottom crust is heated through the tray by conduction. This results in a lesser impact of heat on DNA degradation at the bottom of the bread.

The sample taken in the middle of the bread might be least affected by the heat exposure. However, DNA content of the sample appears to be lower than samples taken just under the top crust. These differences can be explained by the presence of water. The higher moisture content contributes to a lesser DNA content and a higher DNA destruction of the sample in the middle of the bread. Moreover, the extractability of the still available DNA might change through the duration of the baking process due to changes in the microstructure of the bread.

### Influence of the baking process

To evaluate the influence of the baking of bread on the degradation of DNA and the detection of DNA through PCR, samples were taken from dough to bread at various stages of the production process, as shown in Fig. 3. The experimental strategy was again based on two steps: (1) the extraction of total genomic DNA and (2) the amplification of the target DNA sequence by PCR. In this case, PCR amplification of a 600-bp fragment was performed.

Total DNA was isolated from the various samples and subjected to agarose gel electrophoresis. As shown in Fig. 4a, the isolated DNA was degraded to a variable extent for all the doughs. DNA fragments were dominantly in the range of 100–1,000 bp, although larger fragments can also be visualised. The degradation of DNA already noted during the preparation of the doughs can most probably be explained by the action of nucleases present in the doughs, as the temperatures to which the bread was subjected to were not high enough to degrade the DNA present. This can also explain the decrease of the DNA smears further in the dough preparation. After baking, a decrease of the intensity of the

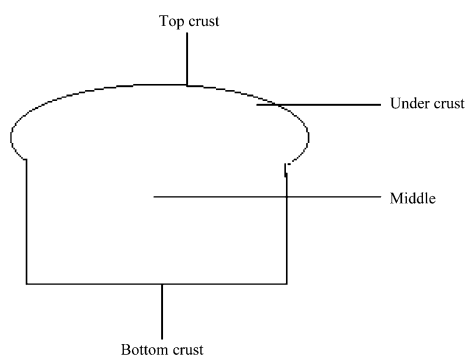


**Fig. 1** Samples taken from a bread for DNA analysis

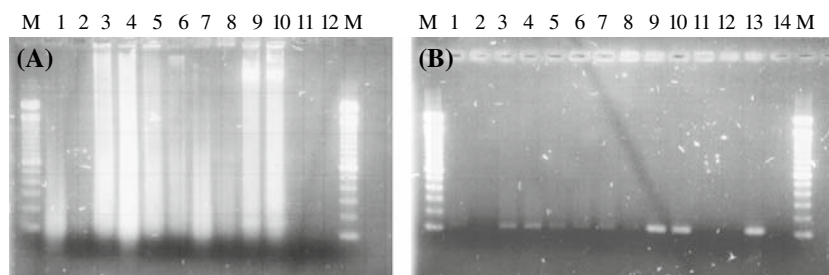


**Fig. 2** Evaluation of DNA from samples taken at different places in bread through (a) agarose gel electrophoresis and (b) PCR analysis. Lanes 1, 2 top crust, lanes 3, 4 under crust, lanes 5, 6 middle, lanes 7,

8 bottom crust, lanes 9, 10 positive extraction control (full-fat soy flour), lanes 11, 12 negative extraction control, lane 13 positive PCR control, lane 14 negative PCR control

**Fig. 3** Samples taken during baking process

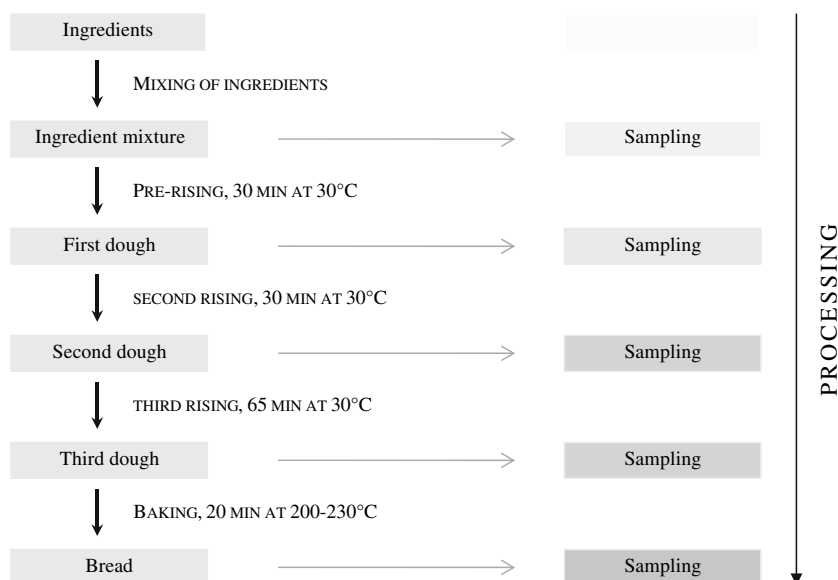
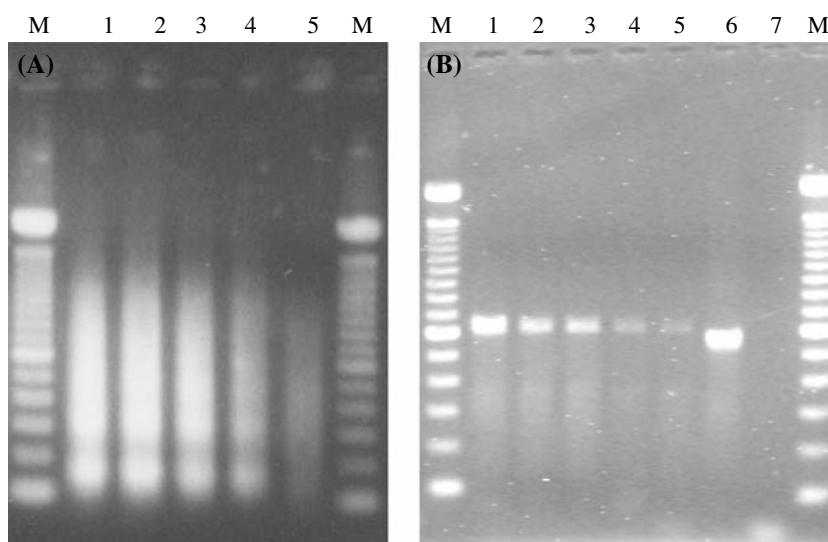


**Fig. 4** Evaluation of the baking process of bread through (a) agarose gel electrophoresis and (b) PCR. Lane 1 ingredient mixture, lane 2 first dough, lane 3 second dough, lane 4 third dough, lane 5 bread, lane 6 positive PCR control, lane 7 negative PCR control



DNA smear is observed, due to the actual baking temperature (200–230 °C). The results in Fig. 4b clearly show that the DNA amplification of a “large” DNA sequence remains

possible, even in the final bread sample. Similar results were obtained by [17] when amplifying a 172-bp fragment and by [10] who amplified a 238-bp fragment in bread.

## DNA from soybean ingredients

DNA was extracted from the soy ingredients and evaluated through agarose gel electrophoresis and PCR detection of the soy lectin gene. Results are shown in Figs. 5, and 6, respectively. Most intense DNA smear is observed for the defatted soybean flour samples and the full-fat flour. High as well as low molecular DNA fragments could be visualised. Clearly, more DNA could be extracted from the full fat than from the defatted flour. This might be explained by a lower extraction efficiency for the full-fat flour due to the fattier matrix and the lower dry matter content. From the toasted soybean flour sample only low molecular weight DNA fragments could be obtained. The heat treatment during toasting reduces DNA fragments to a length below 600 bp. No DNA could be visualised through gel electrophoresis for the soybean fibre sample. For the soy protein isolate sample, nearly no high molecular weight DNA fragments were present, most DNA fragments were smaller than 1,000 bp.

Similar results could be obtained from the spectrophotometrical determination of the concentration of these samples (Table 1). The highest concentration is obtained for the defatted soybean flour samples, with a mean concentration of 866 ng/ $\mu$ l DNA extract. The mean DNA concentration of the full-fat soybean flour is 353 ng/ $\mu$ l. The toasted soybean flour clearly has a lower DNA concentration, 72 ng/ $\mu$ l, whereas the soy protein isolate still contains 144 ng/ $\mu$ l DNA.

Extracted DNA can also be assessed by  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  UV absorption ratios with a spectrophotometer to measure its purity. It is generally agreed that an  $A_{260}/A_{280}$  ratio of 1.8 for DNA is indicative of a pure nucleic acid preparation, i.e. protein free [18, 19]. Absorption at 230 nm reflects contamination of the sample by components such as carbohydrates, peptides, phenols or aromatic compounds.

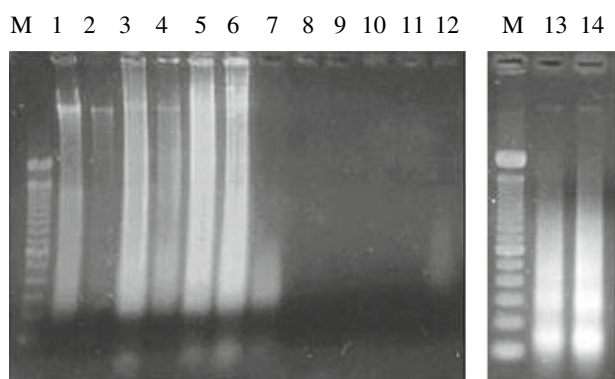


**Fig. 5** Evaluation of soy ingredients through agarose gel electrophoresis. Lanes 1, 2 wheat flour, lanes 3, 4 full fat soybean flour, lanes 5, 6 defatted soybean flour, lanes 7, 12 toasted soybean flour, lanes 8, 9 soy fibre, lanes 10, 11 extraction blanks, lanes 13, 14 soy protein isolate

When the absorption ratio for 260/280 nm is between 1.5 and 2.0, and the absorption for 260/230 nm is more than 1.7, the extracted DNA should be suitable for PCR analysis [18, 19]. No DNA is present in the soy fibre sample. All samples have high  $A_{260}/A_{280}$  values, ranging from 1.60 to 1.87. In other words, all samples should contain DNA of high purity and quality for PCR analysis, except for the soybean fibre, where no DNA could be extracted.

After PCR amplification, positive results can be obtained for the full fat, defatted and toasted flours, and the soy protein isolate (Fig. 6). Although the DNA of the toasted flour appears to be highly degraded, some amplifiable DNA remains present. For the soybean fibre, no amplification for the lectin gene can be obtained. This is supported by the results obtained through agarose gel electrophoresis and spectrophotometry of the samples. The wheat flour sample also results negative for PCR amplification, which means that no soy is present in the wheat flour.

## Practical detection limits

A part of the wheat flour in the bread was exchanged to variable extents with the soy ingredients mentioned above, to determine the influence of the entire baking process on these ingredients. The used amounts, expressed as weight percentage on total dry weight, were 0.2 wt%–0.4 wt%–0.6 wt%–1.0 wt%–2.0 wt%.

The practical detection limit is defined by the amount of ingredient which should be present in a sample to allow a positive amplification [20]. These limits may depend on the PCR method used and have been established for the detection of GM soy in cookies by [13]. Practical detection limits give an idea of the true presence of an ingredient to allow detection, whereas the used technique is based on the actual detection of DNA.

For the defatted as well as for the full-fat soybean flour samples, an amount as low as 0.2 wt% of the soy ingredient on total flour weight basis could be detected. Although less DNA could be extracted from the defatted flour sample, this quantity appeared not to have any effect on the detection in the bread. For the soy protein isolate, an amount of at least 1.0 wt% was necessary to enable positive amplification of the lectin gene. The used amounts of soybean fibre and toasted soybean flour did not allow amplification. Although DNA could be amplified for the toasted soybean flour sample, apparently the amounts used in breads is not high enough to enable PCR detection.

## Commercial flours

Several commercial wheat flours containing soy ingredients were analysed, and these samples were:

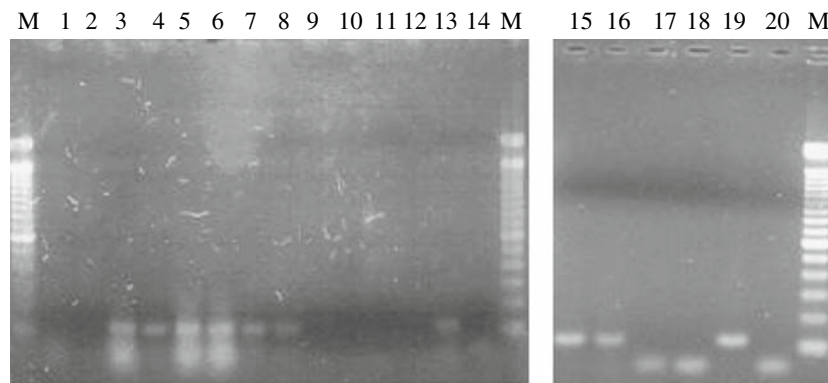


**Fig. 6** Evaluation of wheat soy ingredients through PCR with GMO3/4 primers. *Lanes 1, 2* wheat flour, *lanes 3, 4* full-fat soybean flour, *lanes 5, 6* defatted soybean flour, *lanes 7, 8* toasted soybean flour, *lanes 9, 10* soy fibre, *lanes 11, 12* extraction blanks, *lane 13*

positive PCR control, *lane 14* negative PCR control, *lanes 15, 16* soy protein isolate, *lanes 17, 18* extraction blanks, *lane 19* positive PCR control, *lane 20* negative PCR control

**Table 1** DNA concentration and  $A_{260}/A_{280}$  values of soy samples used is bread

| Sample                 | DNA concentration (ng/ $\mu$ l) | $A_{260}/A_{280}$ | $A_{260}/A_{230}$ |
|------------------------|---------------------------------|-------------------|-------------------|
| Full-fat soybean flour | 429                             | 1.87              | 2.55              |
|                        | 277                             | 1.71              | 2.50              |
| Defatted soybean flour | 921                             | 1.79              | 2.68              |
|                        | 810                             | 1.83              | 2.64              |
| Toasted soybean flour  | 100                             | 1.75              | 2.34              |
|                        | 44                              | 1.80              | 2.00              |
| Soy protein isolate    | 144                             | 1.83              | 1.75              |
|                        | 143                             | 1.63              | 1.76              |
| Soy fibre              | 0                               | –                 | –                 |
|                        | 0                               | –                 | –                 |

- Standardflower: containing 0.2 wt% full-fat (enzyme-active) soybean flour
- Goldflower: containing 0.6 wt% full-fat (enzyme-active) soybean flour
- Panivita flower: 1 wt% full-fat soybean grits
- Santovit: 7.5 wt% full-fat toasted soy flakes and 5 wt% soybean fibre
- Soy meal: 15 wt% full-fat toasted soy flakes.

DNA of these samples was extracted and compared through agarose gel electrophoresis and PCR detection of the lectin gene. Results of the agarose gel electrophoresis did not show huge difference between the different samples, although a lighter smear could be noted for the soy meal sample. Soy contents appear to be high enough to enable a positive amplification for the lectin gene for all samples (data not shown).

The flours were used to make bread and PCR amplification of the lectin gene of these bread samples, were all

positive (data not shown). For the standard flower, containing 0.2 wt% full-fat flour, a positive amplification is obtained, which is in accordance with the bread (see above). For the samples containing toasted soy, santovit and soy meal, clear positive signals can be obtained. Most probably, the amounts of toasted soybean flour used for the determination of the practical detection limits in the previous experiment, i.e. 2.0 wt%, is too low to enable amplification. With a content of 7.5 wt% (santovit) and 15 wt% (soy meal), the amount of toasted soybean flour is much higher and therefore amplification results positive.

## Conclusion

Although DNA is degraded during the baking of bread, PCR detection can still be performed as some DNA remains persistent to the heat treatment. However, in cases where low target DNA is present, due to the fact that the content of the ingredient in the food product is low and/or that the DNA content of that ingredient is low, PCR amplification may become difficult. Therefore, the determination of detection limits, absolute (in terms of copy numbers) or practical (in terms of ingredients), should be well established, because even small amounts of an allergen may lead to an allergenic reaction in sensitive patients. Moreover, in the case of bread, it has been shown that the method of sampling may largely influence PCR results.

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