ISSN 1330-9862 *original scientific paper*
doi: 10.17113/ftb.55.04.17.5192

Threshold Level and Traceability of Roundup Ready® Soybeans in Tofu Production

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Received: February 1, 2017
Accepted: August 2, 2017

Summary

The aim of this study is to assess DNA degradation, DNA amplification, and GMO quantity during tofu production. Soybean seeds were spiked with Roundup Ready® soybeans (RRS) at 0.9, 2, 3 and 5 % (by mass), to assess the level of RSS that would be of practical interest for threshold labelling. Real-time polymerase chain reaction (PCR) was more effective than conventional PCR in the analysis of raw soymilk, okara, boiled soymilk and tofu. The negative effect of grinding and mechanical manipulation was obvious in the okara sample prepared with 3 and 5 % RRS, where GMO content was reduced to (2.28±0.23) and (2.74±0.26) %, respectively. However, heating at 100 °C for 10 min did not cause significant degradation of DNA in all samples. The content of RRS in the final product, tofu, was reduced tenfold during processing, ranging from 0.07 to 0.46 %, which was below the labelling threshold level. The results are discussed in terms of global harmonization of GMO standards, which could have the positive effect on the trade of lightly processed foodstuffs such as tofu, especially regarding the labelling policies.

Key words: DNA degradation, GMO, labelling, legislation, tofu, traceability

Introduction

The production of soyfood has been increasing throughout the world, especially of tofu, tasty and nutritional vegetarian food. Tofu production has changed only slightly over the last 2000 years. It may vary depending on the producer, but the basic steps always include soybean soaking, grinding, soymilk boiling, and adding coagulants. A by-product of soymilk/bean curd production is called okara, a soy residue traditionally used as a food ingredient in Japanese soups, salads and vegetable dishes, or in the feed production (*1*). Transgenic variety of soybeans tolerant to glyphosate (Roundup Ready® soybeans, RRS) is the most commonly cultivated genetically modified crop, which presents approx. 83 % of global soybean production annually and is included in food and feed chain (*2*).

Polymerase chain reaction (PCR) efficiency can largely be affected by food processing. The degradation of DNA during processing can result in over- or underestimation of GMO content (*3,4*). The content of transgenic components is in direct correlation with the degradation degree of endo- and exogenous genes, and it oscillates during food production (*5*). Heating is a commonly used method for food processing, however, it can induce DNA degradation, particularly under severe conditions. The efficiency of GMO determination in processed foods is affected by the degree of degradation of recombinant and taxon-specific DNA sequence (*6*). It is currently not possible to avoid different degrees of degradation in these two DNA sequences and to reliably determine whether the GMO content (in %) in processed foods is in line with the actual values in the raw materials (*7*).

Many studies have dealt with the detection of GMOs in commercially available raw and processed foods in different countries: Brazil (*8*), Canada (*9*), Malaysia (*10*), Serbia (*11*), Hungary (*12*), Turkey (*13,14*) and Portugal (*15*). Moreover, the most recent studies have focused on trac-

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ing DNA or proteins in certain food products during the manufacturing (*16-19*).

There is a high degree of heterogeneity in international legislation concerning traceability and labelling of genetically modified organisms (*20*). In the European Union, GMO regulations established a labelling threshold value of 0.9 % for adventitious or technically unavoidable GMO content. However, threshold value in other countries varies from 0.9 % in Russia to 3 % in South Korea and 5 % in Japan, South Africa, Thailand, Indonesia and Taiwan. Mandatory labelling is common in the East Asian region (*21*). Contrary to this, in the United States, being the largest producer of GMO soybean, voluntary labelling is allowed. These differences in international GMO policies create fragmentation of worldwide markets.

Some studies reported that concentration of transgenic component remained stable during tofu manufacturing, and demonstrated that the procedures of boiling and adding bittern did not cause dramatic DNA degradation (*22,23*). In contrast, others have shown that the thermal treatment affected the exogenous more than the endogenous gene in soymilk processing (*7*) and had extensive effects on transgenic protein degradation (*24*). The discrepancies in the results were mainly attributed to distortions in size of the flour particles used for analysis, differences in the amplicon sizes of target genes and extraction efficiency (*25*).

This study was conducted with soybean seed samples containing different percentage of RRS: 0.9, 2, 3 or 5 % (by mass), which are the legal threshold levels in different countries. The aim of this study is to assess the effect of DNA degradation on the determination of GMO content in soy-containing raw materials and in final tofu products throughout the production. Also, the traceability of transgenic and endogenous DNA in soyfood during tofu production is evaluated. Our results contribute to the knowledge on GMO traceability in lightly processed food and reestablish the acceptable GMO threshold value in the international trade of raw and processed soybean food and feed.

Materials and Methods

Seed material

The samples were prepared in-house by spiking the grounded conventional soybean (*Glycine max* L.) seed (cultivar Vojvođanka; Institute of Field and Vegetable Crops (IFVCNS), Novi Sad, Serbia) with the appropriate amount of soybeans (cultivar Roundup Ready®; Monsanto, Bucharest, Romania). The seeds were ground with Thermomix TM21 (Vorwerk, Wuppertal, Germany) food processor for 2 min at maximum speed. The mass fraction of GM ingredient in the spiked samples was tendentiously set at 0.9, 2, 3 and 5 %. Samples were prepared in two replicates. Before the mechanical manipulation, transgenic content was determined in control materials (0.9, 2, 3 and 5 % RRS).

Food matrix

Soybeans (100 g) from the IFVCNS collection were soaked in 800 mL of distilled water at room temperature for 16 h. The resultant slurry (in a ratio of 1:8, by mass) was then filtered through double-layered cheesecloth to separate soymilk and okara. Soymilk was boiled for 10 min at 100 °C, allowed to cool to about 75 °C as the suitable coagulation temperature, and the coagulant, 2 % $MgSO_4$ (Sigma-Aldrich, Taufkirchen, Germany) was added. It was mixed well and placed in a bath at 75 °C for 10 min. Whey was drained during 2.5 h under the pressure of 1.96 kPa.

Sampling from the experimental production chain included raw soymilk, okara, boiled soymilk and tofu, and was performed in three replicates for each product.

DNA extraction

DNA was extracted from 100 mg of the samples using peqGOLD Plant DNA Mini Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany), according to the manufacturer's instructions. The concentration of DNA in the extracts was estimated using a spectrophotometer (Genesys™ 10S UV-Vis; Thermo Scientific, Madison, WI, USA). The quality of extracted DNA was further analyzed by electrophoresis on 1 % agarose gel (Serva Electrophoresis GmbH, Heidelberg, Germany) containing ethidium bromide (0.5 g/mL; Sigma-Aldrich). The agarose gel was visualized under UV light and a digital image was obtained using a Doc-Print system (Vilber Lourmat, Eberhardzell, Germany).

European reference materials (ERM), *i.e.* ERM-BF410® with 0, 0.1, 0.5, 1, 2 and 5 % RRS content (Institute for Reference Material and Measurements (IRMM), Joint Research Centre, Ispra, Italy), were used in the experiments as positive and negative controls.

Qualitative PCR

In order to detect amplifiable DNA in all samples, we firstly used primer pair for lectin gene: GMO3 and GMO4, which amplifies a fragment of 118 bp (*24,25*). The event-specific PCR was performed using primers 35s-f2 and petu-r1 (Metabion International AG, München, Germany), which amplify a fragment of 172 bp (*26*).

PCR was performed with 2× PCR Master Mix (Fermentas, Vilnius, Lithuania) containing 4 mM $MgCl_2$, 0.4 mM dNTP and 0.05 units per μL of Taq DNA polymerase (recombinant) in a final volume of 25 μL, containing 0.2 pmol/μL of primers for both lectin and RRS genes, and 50 ng of DNA.

Amplifications were carried out in a Mastercycler® ep Gradient S Thermal Cycler (Eppendorf, Hamburg, Germany) with the following program: denaturation at 94 °C for 10 min, followed by 30 cycles at 94 °C for 30 s, 63 °C for 30 s and 72 °C for 30 s (for lectin gene); 35 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s (for RRS gene), and the final extension was carried out at 72 °C for 3 min. Four extracts of each sample from the production chain were amplified in the assays. Positive and negative controls (0, 0.1, 1 and 2 % ERM RRS) and blank control were included in each run.

The amplification fragments were determined using electrophoresis on 2 % agarose gel containing 0.5 g/mL of ethidium bromide (Sigma-Aldrich). A FastRuler™ Low Range DNA Ladder (SM1103, Fermentas, Vilnius, Lithuania) was used as a marker.

Real-time PCR

DNA quantification was performed using 7300 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) in 96-well microtiter plates with a total volume of 20 μL. The analytical method applied was a RRS construct-specific method: the soybean-specific primers amplify an 81-bp fragment of the lectin gene and the RRS primers amplify an 83-bp fragment of the transgenic chloroplast transit peptide (CTP) from *Petunia hybrida* to a 35S promoter (*13,27*).

Temperature program included initial denaturation for 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 min and 72 °C for 31 s.

Data analysis of qPCR results

After the run was completed, the data were analyzed using the ABI PRISM 7700 SDS software in order to obtain cycle threshold (Ct) values of each reporter dye (FAM™ and VIC® fluorescence dyes) for each sample (*28*). The threshold values were adjusted for the FAM and VIC dye layers. After analyzing the run, the results were exported to a file in GMO Analysis Excel Macro 1.7 file provided by manufacturer (Applied Biosystems, Foster City, CA, USA). The Ct(FAM) and Ct(VIC) average values were calculated for each group of replicates in order to calculate the ΔCt values (Ct(FAM)–Ct(VIC)). For each sample, the content of GMO was calculated by comparing the ΔCt of the sample to the set of logarithmic values of GMO content (in %) and ΔCt values obtained from the standard set of RRS (*i.e.* ERM-BF410 series with 0, 0.1, 1 and 10 % RRS content).

Statistical analysis

The results were compared by one-way analysis of variance (ANOVA). All data were expressed as mean values±standard deviation.

Results and Discussion

Concentration and quality of extracted DNA

The concentrations of the extracted DNA, calculated according to the average value of all performed extractions per product, ranged from approx. 12 ng/μL in raw and boiled soymilk to 147.7 ng/μL in tofu (Table 1). Absorbance ratios at 260 nm/280 nm and 260 nm/230 nm were used as DNA purity parameters (*29*). The average ratios of $A_{260\,nm}/A_{280\,nm}$ were similar for all samples, around 1.8, and they indicate low protein impurities. Low $A_{260\,nm}/A_{230\,nm}$ ratios measured in soymilk and okara indicate the presence of carbohydrates.

Table 1. Concentration and purity of DNA preparations extracted in four steps of tofu production

Sample	γ(DNA)/(ng/μL)	$A_{260\,nm}/A_{280\,nm}$	$A_{260\,nm}/A_{230\,nm}$
Raw soymilk	11.7±5.1	1.79±0.14	1.35±0.80
Okara	36.7±6.8	1.72±0.14	0.94±0.34
Boiled soymilk	12.0±3.8	1.82±0.12	1.39±0.76
Tofu	147.7±68.7	1.92±0.03	2.29±0.24

Values are mean of four reactions per each threshold level±standard deviation

Qualitative PCR

The presence of soybean DNA in the samples and its quality was checked by using soybean-specific primers for lectin gene. From several primers available in the literature one primer set was chosen, amplifying a short fragment of 118 bp (GMO3/GMO4). The fragments of 118 bp (Fig. 1), corresponding to a part of the endogenous lectin gene, were amplified in all the samples. The results are in agreement with findings that lectin gene was detectable in natto if the primer pair with the expected band size of 118 bp was used (*30*).

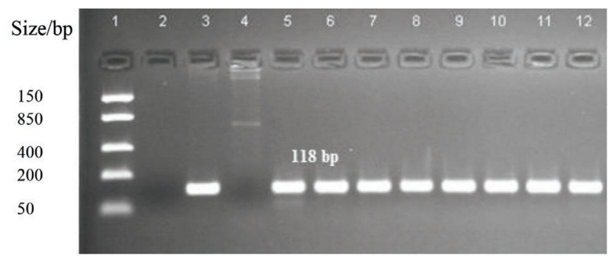

Fig. 1. Detection of the soybean lectin gene using GMO3/GMO4 during tofu production from soybeans with 0.9 % Roundup Ready® soybean (RRS). Lane 1=DNA ladder, 2=blank, 3=soybean (positive control), 4=negative control, 5 and 6=raw soymilk, 7 and 8=okara, 9 and 10=boiled soymilk, 11 and 12=tofu

Qualitative detection is the first critical step in GMO analysis since only positive samples detected during the screening are further identified and quantified (*31*). The screening was performed for the presence of event-specific RRS, and it was expected that at least samples derived from starting material with high percentage of RRS would produce a fragment of 172 bp as a result of soybean *epsps* gene amplification. Amplification was achieved in all samples of okara and boiled soymilk or several raw soymilk samples (Table 2). There was no amplification of *epsps* gene in tofu samples. The sensitivity of PCR reaction was checked using three controls, 0.1, 1 and 2 % RRS ERM, which gave a visible band (Fig. 2). These results imply that a fragment of 172 bp from *epsps* gene could not be detected in tofu, probably due to cleavage of the of *epsps* gene.

Quantitative PCR

The transgenic content was detected by real-time PCR in all samples. DNA degradation induced by processing was evaluated by employing qPCR assays that allowed simultaneous detection of RRS gene, as well as soybean-specific lectin gene in RRS construct-specific reaction. PCR efficiency and coefficient of determination (R^2) of qPCR calibration curves were identified as parameters of reliable determination of GMO content (*32*). Both standard curves showed a high degree of correlation ($R^2 \geq 0.998$) in all the assays for the *epsps* and lectin genes, while PCR efficiencies ranged from 99 to 101 %, indicating the convenience of the standard curves for quantification. The standard deviation

Table 2. The results of PCR and real-time PCR amplification of *epsps* gene in four steps of tofu production

Sample	w(RRS)/%							
	0.9		2		3		5	
	PCR	qPCR/%	PCR	qPCR/%	PCR	qPCR/%	PCR	qPCR/%
Raw soymilk	+	0.05±0.04	+	0.15±0.04	-	0.24±0.12	+	0.32±0.17
Okara	+	0.82±0.10	+	2.21±0.53	+	2.28±0.23	+	2.74±0.26
Boiled soymilk	+	0.07±0.02	+	0.17±0.05	-	0.25±0.07	+	0.45±0.09
Tofu	-	0.07±0.01	-	0.24±0.03	-	0.27±0.06	-	0.46±0.19

Values are mean of four reactions per each threshold level±standard deviation. PCR=polymerase chain reaction, qPCR=quantitative PCR

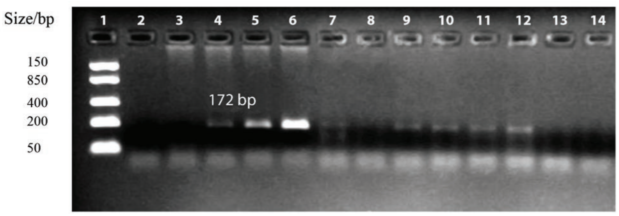

Fig. 2. Analysis of the presence of *epsps* gene during tofu production from soybeans with 0.9 % Roundup Ready® soybean (RRS). Lane 1=DNA ladder, 2=blank, 3=0 % RRS ERM (negative control), 4=0.1 % RRS ERM (positive control), 5=1 % RRS ERM (positive control), 6=2 % RRS ERM (positive control), 7 and 8=raw soymilk, 9 and 10=okara, 11 and 12=boiled soymilk, 13 and 14=tofu. ERM=European reference material

between replicates ranged from 0.01 to 0.23. In this study, 10 min of heating at 100 °C did not cause significant degradation of DNA. The transgenic content with estimated real-time PCR in raw and soymilk after boiling was similar for all four levels of GMO (Table 2). The increased content of Roundup Ready® Soybean RRS gene in milk with 5 % of RRS after boiling (from (0.32±0.17) to (0.45±0.09) %) could not be explained as a result of degradation of endogenous DNA (Fig. 1 and Table 3). Our results confirmed that the lectin sequence in transgenic food was more stable than *cp4epsps* sequence (*33*). Degradation of PCR target mainly depends on length, GC content and localization on the chromosome (*7*). The native protein could not be detected after dry heating for 10 min, thus confirming the decomposition of the mature protein (*17*). Increasing the time of heating for 30 min led to the recovery of only 43 % of RRS gene and 43 % of the endogenous gene target sequences (*34*).

Table 3. The cycle threshold (Ct) values of lectin gene in four steps of tofu production

w(RRS in seed)/%	Ct			
	Raw soymilk	Okara	Boiled soymilk	Tofu
0.9	27.20±0.80	27.23±0.08	27.25±0.33	28.90±0.67
2	27.09±0.46	27.67±0.58	27.39±0.52	26.45±0.38
3	27.47±0.49	25.89±0.39	28.02±0.55	26.69±0.09
5	27.79±0.52	26.74±0.26	28.04±0.75	27.04±0.45

Values are mean of four reactions per each threshold level±standard deviation

The negative effect of grinding and mechanical manipulation was obvious in the okara sample prepared with 5 and 3 % RRS, where the content of GMO was reduced to (2.74±0.26) and (2.28±0.23) %, respectively (Fig. 3). As a result, the exogenous gene was more damaged than the endogenous gene (Table 3), while the content of transgenic components rapidly decreased. The results are in agreement with the previous findings, which indicated that mechanical treatment such as grinding resulted in degradation of exogenous and endogenous genes during soymilk processing (*6*). In Japan, the threshold value of 5 % GMO is set for food, but not for livestock feed, so it can be concluded that okara can be used in feed preparations without labelling (*35*).

The transgenic content was more or less in accordance with the reference material in okara samples derived from the samples with lower content of RRS (2 and 0.9 %). Several studies have reported that mechanical treatment does not significantly affect the degradation of endogenous or exogenous genes (*21*).

It has been reported that the relative amount of genetically modified content does not change with food processing (*36*). The quantitative tests are applied to shorter

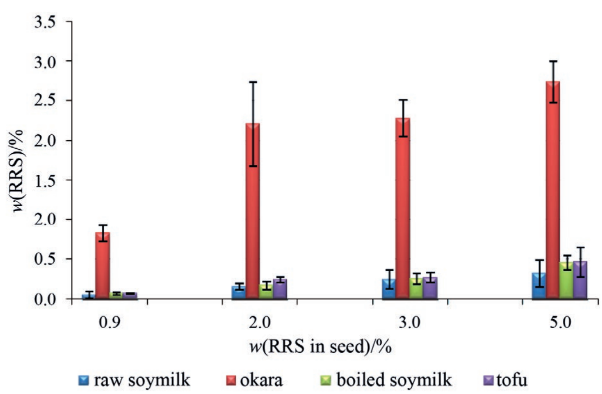

Fig. 3. Real-time results of the amplification of *epsps* gene during tofu production. Bars represent standard deviations. RRS=Roundup Ready® soybean

amplicons and when the amplicon size of the transgenic and taxon-specific gene is similar (*36*), as was the case in this study.

The particle size affects the extractability of the DNA. Smaller particles of the food matrix have better extractability, while high-molecular-mass DNA, at the beginning of heat treatment, is degraded differently (*36*). The content of RRS in boiled soymilk was almost the same as in raw soymilk. The increased transgenic content was obtained in boiled soymilk, prepared with 5 % RRS. The content of RSS in boiled soymilk and tofu was also similar. Slight change in pH (from 6.3 to 5.7) caused by adding coagulant (2 % $MgSO_4$) to soymilk could additionally affect DNA degradation in tofu. Coagulation with salt occurred during cross-linking of cation Mg^{2+} with protein molecules in soymilk, which decreases protein solubility to form curd (*37*). The content of RRS in tofu was reduced approx. tenfold compared to the starting raw soybean material and ranged from (0.07±0.19) to (0.46±0.19) %. Our results confirmed the reports stating that boiling and adding bittern did not cause dramatic DNA degradation (*22*). Thermal treatment had a relatively more dramatic effect on the exogenous gene than the endogenous one (*5*) and our results are in agreement with that. The degree of DNA degradation in a specific food product can be predicted if the processing parameters are available (*38*).

The GMO content in processed materials may not always reflect the actual GMO content in the raw material. The Japanese government requires mandatory labelling when GMO material is present in the top three raw ingredients and accounts for 5 % or more of the total mass (*38*), while the EU regulations require labelling of the foods that contain 0.9 % or more of GMO DNA or protein. Starting with labelled raw soybean, GMO content in the final product, tofu, was below the labelling threshold during processing.

The GMO content of 0.9-5 % in starting soybean seed slurry was reduced in all intermediate and final tofu products manufactured in this study. Okara and tofu products with starting seed slurry containing above 0.9 % GMO exceeded the labelling threshold level according to the EU, but not according to Japanese legislative (*35*). This result shows that the process of global harmonization of GMO legislation would have a considerably positive trade effect on lightly processed foods such as tofu, especially with regard to labelling policies.

Conclusions

Many steps of food processing affect the state of the DNA. The grinding, mechanical manipulation and thermal treatment had more effect on the degradation of DNA during tofu production than boiling and addition of bittern. GMO gene was more affected by these treatments than the exogenous gene. The effect of DNA degradation on GMO determination was evident in the final tofu products. The content of Roundup Ready® soybean in the tofu produced using mechanical treatment was reduced below the different international threshold labelling levels (from 0.9 to 5 %). Based on these results, it could be concluded that the traceability strategy of all GMO in the food production system requires a completely new labelling system. These data represent an important accomplishment for promoting a uniform traceability system of GMOs at the international level, affecting reestablishment of the threshold level for raw material, food and feed products.

Acknowledgements

This study was supported by the Project no. TR31024 of the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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