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GENETICALLY MODIFIED SOY IN LOCAL TOFU: EFFECT OF TOFU PREPARATION ON TRANSGENIC ELEMENT *EPSPS* (5-ENOLPYRUVYLSHIKIMATE-3-PHOSPHATE SYNTHASE)

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ABSTRACT

Tofu (soybean curd) and its derivatives are not only the principal protein component of meals for Asians and vegetarians but are also a popular side dish for Westerners because it is a good source of proteins. However, consumption of tofu may now have a perceived risk because the tofu may be contaminated with transgenic elements such as the cauliflower mosaic virus (CaMV) 35S promoter, *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) and the nopalin synthase (*nos*) terminator. Although no evidence has been found for an effect of the contaminated elements in tofu on human health, and food processing degrades the DNA, consumers still worry. So, to relieve apprehension of the consumers, tofu from markets in Bangkok and the vicinity at Bangkok were randomly sampled and examined for the transgenes by PCR. Genetically modified soybeans were also analyzed to study the effect of processing steps on degradation of *EPSPS* during the process of tofu making. During tofu processing, samples were taken from each step to analyse for the quantity of *EPSPS* by PCR-ELISA. Results indicate 3 out of 106 samples (ca. 2.8%) were contaminated with CaMV 35S promoter, *nos* terminator DNA fragments and *EPSPS*. In addition, *EPSPS* content in tofu prepared from GM soybeans was not affected by the tofu making process.

KEYWORDS: Tofu, transgenes, *EPSPS*, tofu processing step

1. INTRODUCTION

Tofu (soybean curd) is a soft to firm cake-like soybean product made by coagulation of soymilk with salts and pressing the resulting curds into blocks within cheesecloth bags using stones or wooden planks to remove the excess water. It originated in ancient China and was a commonly produced and consumed food item in China by at least the 2nd century BC [1]. Subsequently, tofu and its production technique were introduced into Japan in the Nara period (late eighth century) as well as other parts of East Asia. Since then, tofu has become a staple part of the diet in many countries, including Vietnam, Thailand, and Korea, with subtle regional variations in production methods, texture, flavor, and usage [2]. It is not known when tofu was introduced into Thailand but it is assumed that tofu was introduced since the Chinese immigration into Thailand.

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Tofu is not only a principal dietary component for Asians and vegetarians but it also has become a popular product to Westerners because tofu is a good source of proteins and carbohydrates and is low in fat but rich in mineral contents. The incorporation of tofu into Western diet could be an important means of preventing and treating chronic diseases such as certain cancers and cardiovascular diseases [3-4]. There are a wide variety of tofu products available in both Western and Asian markets. Despite the daunting variety, tofu products can be split into two main categories: fresh tofu, which is produced directly from soy milk, and processed tofu, which is produced from fresh tofu. Tofu production also creates important side products which are often used in various cuisines.

Due to the high consumption of tofu and its derivatives as well as other soya products such as soya milk and the bean itself in the world, the demand for soybeans has increased continuously. Thailand is not a major soybean producing country but is one of the major soybean consuming countries and thus raw soy grain has to be imported into Thailand. Thus, GM soybean may potentially appear in the markets from elsewhere. GM soybean has been developed by transformation of the non-transgenic soybean with the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene of *Agrobacterium tumefacien* strain CP4 linked to other transcription and control elements, the cauliflower mosaic virus (CaMV) 35S promoter and the nopalin synthase (*nos*) terminator, into the plant genome. The plant carrying *EPSPS* exhibits resistance to Roundup glyphosate herbicide [5]. Because ordinary and GM soybean look the same, tofu producers could unintentionally use GM soybeans instead of ordinary soybean for making tofu. Although no evidence has been presented to date that the *EPSPS* and its products have any harmful effect on the human health. Some people fear that introduced genes may provoke allerginicity and gastrointestinal problem such as the case of the Brazil nut-soybean [6]. Food derived from GM plants may cause antibiotic resistance. An antibiotic resistance gene used as a marker gene in transgenic plants may naturally flow to gut bacteria. GM food could also be possibly altered expression of macro- and micro-nutrients or the formation of novel toxins [7]. With these reasons, some people totally oppose any form GM food. Thus, tofu and other soya products are considered as high risk for genetically modified materials contamination and their use as a food source has raised public safety concerns. To address food safety concerns, Thai government do not allow to cultivate any GM plants commercially and to use GM plants for food. Labeling regulation (5% threshold level) is also applied to GM food.

It has also been reported that food processing may lead to partial or complete degradation or removal of the DNA [8], it is doubtful whether physical treatments such as griding and boiling steps in the process of tofu making would affect the degradation of transgenetic materials such as *EPSPS* present in GM soybean. The CP4 *EPSPS* was selected as a marker gene to study the effect of processing because this gene is the major transgene in GM plants and encoded the EPSPS enzyme which is part of the shikimate pathway and involved in the production of aromatic amino acids and other aromatic compounds in plants [9]. In addition, the Certified Reference Materials (CRM) standards of GM soybean is also available for quantitative analysis. The aims of this report were to study (i) the contamination of *EPSPS* in the tofu markets in Bangkok and surrounding vicinities and (ii) the effect of processing steps on degradation of *EPSPS* during the process of tofu making.

2. MATERIALS AND METHODS

2.1 Materials

Reference standard

The Roundup Ready™ (Monsanto, USA) certified reference materials (CRM) of genetically modified dried soybean powder with 0%, 0.1%, 0.5%, 1%, 2% and 5% (w/w) were purchased from Fluka and used as positive and negative controls for the detection analysis.

2.2 Methods

2.2.1 Tofu sampling

A total of 106 tofu samples each of 10 x 10 x 3 cm in size were randomly collected from various local fresh markets in Bangkok and the surrounding areas between November 2004 – January 2005.

2.2.2 Genetically modified tofu preparation and sample collection

Raw GM soy grain used for tofu preparation was kindly provided by the Department of Agriculture (DOA). Tofu was made as follow: Raw GM soy grains (800 g) were first washed and then left to absorb tap water for about 3-4 hours and then ground in 5 liters of water with an electric mill to form a ground soybean paste (go). This was filtered with white cotton cloth and squeezed out to obtain soymilk. The soymilk was boiled for 10 min, cooled to 70°C – 80°C and $MgSO_4$ solution {2 ml at a concentration of 3.8 g/ml (w/v)} was gently and thoroughly mixed into the soymilk. Once the soymilk became coagulated, it was poured into a wooden molding box. Tofu (called "Momen-tofu) was prepared by removing the fluid from the coagulation with a weight (500 g) for about 20 min. Tofu intermediate samples for DNA extraction were collected as illustrated in Figure 1 during the procedure.

2.2.3 Genomic DNA extraction

Each sample, following the addition of 100 ml sterile distilled water, was homogenized with a blender and then centrifuged at 12,000 rpm for 10 min. The supernatant was decanted. A portion of (500 mg) the homogenate was transferred to 2.0 ml sterile eppendroff tube followed by addition of 1.6 ml DNA extraction buffer containing 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA and 1% SDS (w/v). Genomic DNA was extracted by guanidine – chloroform method [10].

Genomic DNA of soy samples at each stage of tofu making was also extracted according to the protocol [10]. Samples taken at stages 1, 3 and 6 were ground before DNA extraction. Samples taken at stages 2, 4 and 5 were centrifuged for precipitation and precipitates were then extracted using guanidinium-chloroform. The quantity and quality of the extracted DNA were monitored with a GeneQuant II RNA/DNA Calculator (Pharmacia) based on absorptions of OD_{260}. DNA concentration of all samples were spectrophotometrically adjusted to 50 ng/μl.

2.2.4 Qualitative PCR assay for *lectin* gene, CaMV 35S promoter and *nos* terminator sequence

Prior to quantitative analysis of *EPSPS* in the samples, the quality of the extracted DNA samples and PCR conditions were evaluated using an assay of the soybean intrinsic gene (*lectin*), CaMV 35S promoter and *nos* terminator sequence using three pairs of specific primers as shown in Table 1

PCR was carried out in reaction mixtures (50 μl) containing 5.0 μl of 10x PCR buffer, 1.0 μl of 10 mM dNTP, 3 μl of 25 mM $MgCl_2$, 1 μl primers with 0.5 μM each, 0.25 μl of Taq DNA Polymerase (Promega) and 10 μl of sample DNA . GM and Non GM soybean DNA and sterile deionized (DI) water were also used as positive and negative controls, respectively. Amplification was performed with a Perkin-Elmer (Gene Amp PCR system 9700) thermal cycler according to the following PCR step-cycle program: pre-denaturation of 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing for 20 s and extension at 72°C for 1 min. A final extension at 72°C for 10 min followed the final cycle for complete synthesis of elongated DNA molecules. The annealing temperature in the cycle program was changed according to the primers used as shown in Table 1. The PCR products were analyzed by electrophoresis (BIO-RAD sub-cell®GT) on a 1.0% (w/v) agarose gel (SeaKem®).

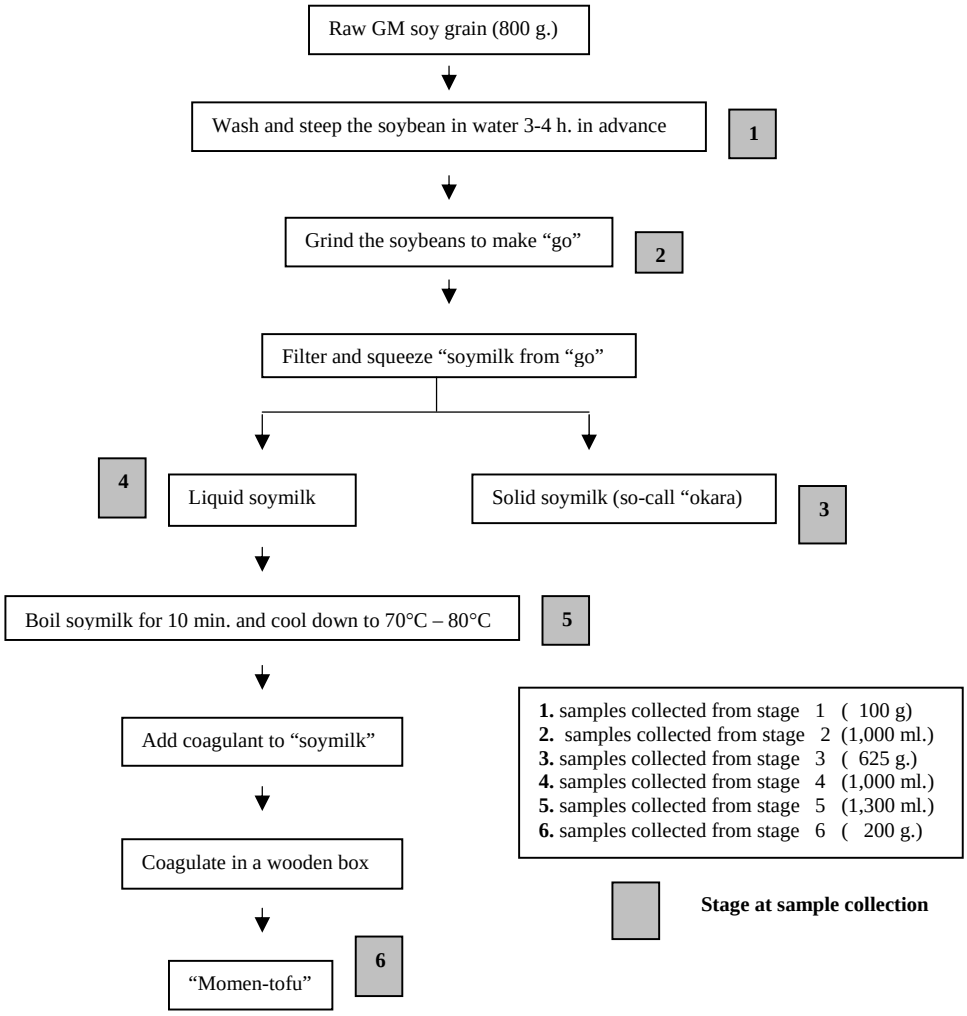


Figure 1 The procedure for making tofu.

Table 1 Primer pairs and sequences used in this study with annealing temperatures and amplicon Sizes

Target DNA fragment	Primers	Primer sequence*	Annealing T (°C)	Amplicon length (bp)
lectin	Lec1	5'-GACGCTATTGTGAGCTCCTC-3'	57°	181
	Lec2	5'-TGTCAGGGCCATAGAAGGTG-3'		
CaMV 35S promoter	35SF2	5'-GCTCCTACAAATGCCATCA-3'	60°	195
	35SR2	5'-GATAGTGGGATTGTGCGTCA-3'		
nos terminator	Nos1	5'-GAATCCTGTTGCCGGTCTTG-3'	54°	180
	Nos2	5'-TTATCCTAGTTTGCGCGCTA-3'		
EPSPS	RRF	5'-TGATGTGATATCTCCACTGACG-3'	60°	172
	RRR	5'-TGTATCCCTTGAGCCATGTTGT-3'		

***** derived from Lipp *et al.* [11]

2.2.5 Semi-quantitatively analysis of *EPSPS* in the samples by PCR-ELISA

2.2.5.1 Detection of digoxigenin-labeled PCR products with PCR-ELISA

DNA of samples and DNA from GM soybean used as positive control and Non GM soybean used as negative control were labeled with digoxigenin using PCR DIG labeling and detection kit (Roche Diagnostics), while sterile DI water was also included as negative control but was not labeled. DIG-labeled PCR were performed according to manufacturer's protocol (Roche Diagnostics) using the RRF (5'-TGATGTGATATCTCCACTGACG-3') as forward primer and RRR (5'-TGTATCCCTTGAGCC ATGTTGT-3') as reverse primer with 60°C annealing temperature. DIG-labeled PCR products were detected using a solution hybridization based microtitre plate assay. The PCR-ELISA was carried out according to manufacturer's protocol (Roche Diagnostics) as briefly described; DIG-labeled PCR products were denatured and hybridized to a streptavidin coated microtitre plate using a specific biotinylated oligonucleotide capture probe. After binding of an antidigoxigenin antibody labeled with peroxidase to the amplified DNA, the ELISA reaction was visualized by adding 2, 2'-azino-di-(ethylbenzthiazolin) sulphonate (ABTS), and the optical density of the color reaction from both samples and control was subsequently measured in a microplate reader at 405 nm.

2.2.5.2 Construction of standard curve

Standard curve for determining the content of *EPSPS* in the samples was constructed in the range of 0%- 5% *EPSPS* by PCR-ELISA. DNA from Roundup Ready™ (Monsanto, USA) certified reference materials (CRM) of genetically modified dried soybean powder with 0%, 0.1%, 0.5%, 1%, 2% and 5% (w/w) were extracted with guanidinium-chloroform and purified by Wizard®Miniprep DNA Purification Kit (Promega., USA). Each purified DNA standards was spectophotometrically adjusted to 50 ng/µl and subsequently used as DNA templates for PCR-ELISA as previously described in 2.2.5.1. The standard curve was obtained by plotting absorbance values versus the percent content of *EPSPS* in CRM soybean powder. A representative standard curve was shown in Figure 2.

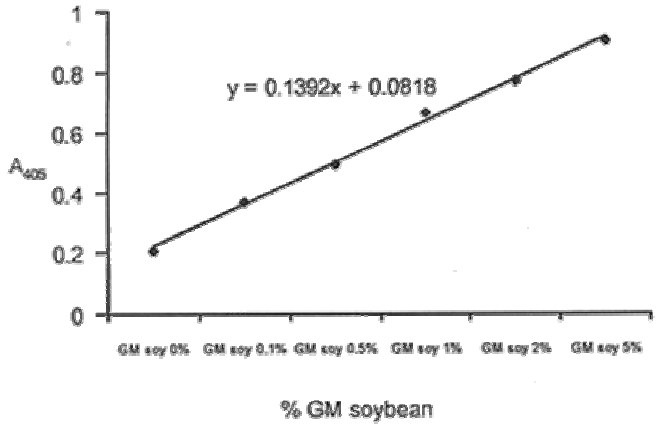


Figure 2 Standard curve for determination of *EPSPS* content in DNA samples taken at different stages in tofu making process

2.2.5.3 Determination of *EPSPS* content in DNA samples

The amount of *EPSPS* content in DNA samples taken at different stages in the tofu making process was calculated by substitution of the absorbance values in the formula shown in Figure 2. Average means from four replicates of each sample were compared by *F*-test ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Genomic DNA extraction from tofu

The genomic DNA was extracted from each tofu sample by the guanidine–chloroform method. The DNA concentration measured by the spectrophotometer with the OD_{260} was variable. It was in the range 15 – 249 ng/μl (average 110.6 ng/μl) with an OD_{260}/OD_{280} ratio of the samples ranging from 1.0-1.7 (average 1.3) (data not shown). The quality of genomic DNA of each tofu sample was also examined on 1% (w/v) agarose gel electrophoresis (Figure 3). In general, the quantity and quality of the genomic DNA were quite high according to spectrophometric analysis, but electrophoretic analysis revealed that most of DNA samples were degraded whilst a few DNA samples were partially sheared as indicated with circles in Figure 3. The results from both analysis showed very different DNA quality. For example, the OD_{260}/OD_{280} ratio of the sample code number 21/4 was 1.721 indicating high quality (data not shown), while the DNA band of the same sample as shown in the lane 93 (Figure 3) showed smearing. Quite often the quality of extracted DNA examined either by the spectrophotometer or agarose gel electrophoresis may not be reliable and correlated [12]. Further analysis of genomic DNA by PCR was necessary to determine the quality of DNA.

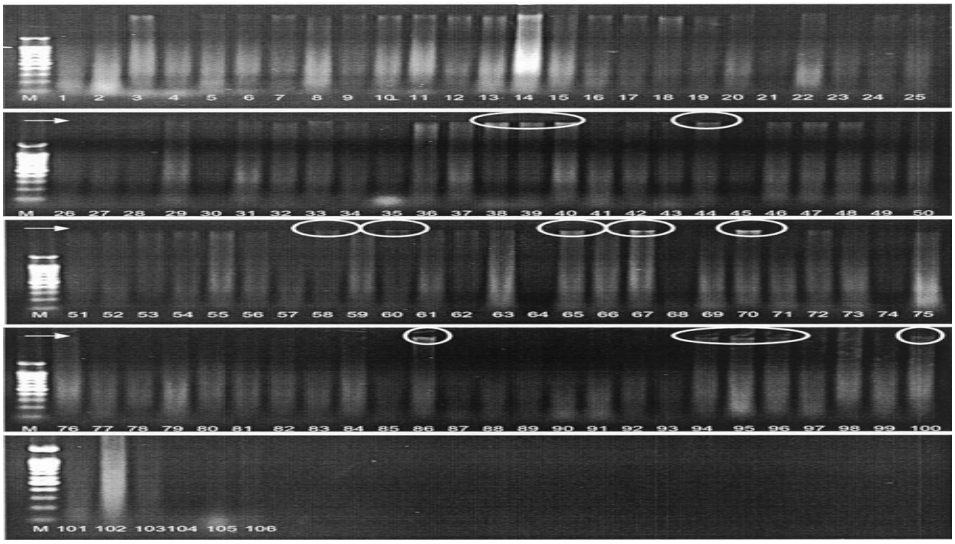


Figure 3 Agarose gel electrophoresis of genomic DNA extracted from each of 106 tofu samples: Lane M, 100 bp DNA Ladder; Lanes 1-106 represent genomic DNA of tofu samples 1 to 106.

3.2 PCR analysis of tofu samples for GM soybean contamination

The GM soybean (Roundup Ready Soybean, *RRS*) contains four inserted elements; the cauliflower mosaic virus (CaMV) 35S promoter, the nopalin synthase (*nos*) terminator, the CTP (petunia derived cell transit peptide sequence) and the CP4-*EPSPS* with the house-keeping gene (*lectin*) [13]. To determine whether the Roundup Ready soybean contaminated into tofu, amplification of three GMO-specific sequences to screen for the presence of transgenic material in tofu was performed using the specific primers shown in Table 1. The quality of DNA and the PCR conditions were also assayed before screening. As shown in Figure 3, although most of the DNA was degraded, the DNA band (181 bp) of the partial *lectin* gene could be amplified (Figure 4).

This indicated that the purity and the integrity of the DNA extraction were suitable for small amplicon PCR.

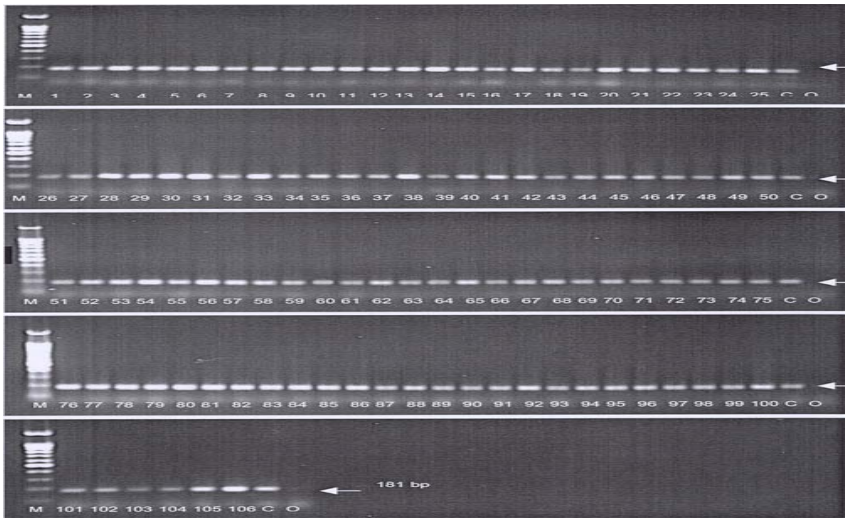


Figure 4 PCR amplification of *lectin* (181 bp) from genomic DNA extracted from each of 106 tofu samples: Lane M, 100 bp DNA Ladder; Lane C, GM soy DNA; Lane O, sterile DI water Lanes 1-106 represent genomic DNA of tofu samples 1 to 106.

Results in Table 2 show that only 3 out of 106 (2.8%) tofu samples were contaminated with the Roundup Ready soybean. Of the 3 contaminated samples, 2 samples were from markets near the railway station. Presumably, the tofu producer in these markets might have unintentionally used GM soy grain from sources outside of Bangkok, e.g. those derived from the animal feed factory or in other provinces and transported by train into the Bangkok market.

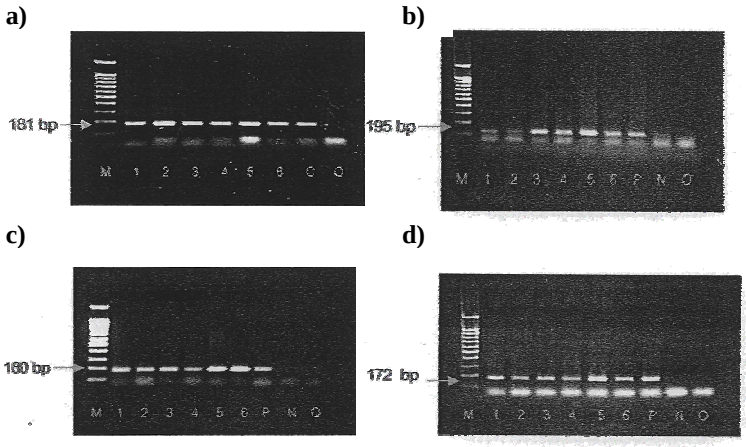
3.3 Effect of tofu processing treatments on the degradation of *EPSPS* in the GM soy grain

To study the effect of tofu processing treatments on the degradation of *EPSPS*, tofu containing GM soy grain was experimentally produced. Qualitative PCR was initially performed to check the PCR conditions and the purity and integrity of the DNA samples extracted from each step. The *EPSPS* content from the initial Roundup Ready raw soy grain, intermediate products and the final products (tofu) was examined to ascertain whether the *EPSPS* content would be changed by the tofu processing steps. Qualitative PCR was found to detect *lectin* gene and three transgenes; the CaMV 35S promoter, the *EPSPS* (*RRS*) and the *nos* terminator in all DNA samples (Figure 5). This figure indicated that tofu processing treatments such as grinding and heating had no impact upon DNA breakdown to below detecting threshold of a 100 bp fragment, although Kharazami *et al*. [14] found that a lot of genomic DNAs were broken down during the grinding step in their experiment. Semi-quantitation of *EPSPS* content was then performed and results indicate that there was no difference in the *EPSPS* content of all samples (Table 3). Similar results were also reported by Moriuchi *et al*. [15] on the study of *RRS* content in processed foods such as tofu made from soy containing *RRS*. However, our experiment was only performed on a small-scale when compared to Moriuchi *et al*. [15]. This result needs to be confirmed.

Table 2 PCR analysis of each transgenic elements contaminated in tofu samples randomly collected from various local fresh markets in Bangkok and vicinity areas around Bangkok between November 2004 – January 2005.

Name of sub districts / districts in Bangkok at tofu samples collected	PCR analysis		
	CaMV 35 S	*nos*	*EPSPS*
Bang sue	0/10 [a]	0/10	0/10
Pravet	0/6	0/6	0/6
Minburi	0/4	0/4	0/4
Bangkapi	0/5	0/5	0/5
Dusit	1/4	1/4	1/4
Chatujak	0/8	0/8	0/8
Pomprapsatupai	1/2	1/2	1/2
Donmuang	0/4	0/4	0/4
Bangkoknoi	1/3	1/3	1/3
Ratburana	0/3	0/3	0/3
Dindang	0/3	0/3	0/3
Huakuang	0/5	0/5	0/5
Sampuntawong	0/5	0/5	0/5
Phatumwon	0/3	0/3	0/3
Laopraw	0/2	0/2	0/2
Khlongtae	0/4	0/4	0/4
Watana	0/2	0/2	0/2
Suunluang	0/2	0/2	0/2
Bangkaen	0/5	0/5	0/5
Bangkae	0/4	0/4	0/4
Pranakorn	0/4	0/4	0/4
Khlongsan	0/4	0/4	0/4
Samutprakarn	0/9	0/9	0/9
PrathumThani (Rangsit)	0/4	0/4	0/4

[a] the number of GM tofus/ the number of tofus examined



Lane M = 100 bp DNA marker
Lane O = control (sterile DI water)
Lane 1 = sample collected at stage 1
Lane 3 = sample collected at stage 3
Lane 5 = sample collected at stage 5
Lane P = Positive control (soybean GM DNA)
Lane N = Negative control (soybean non-GM DNA)
Lane 2 = sample collected at stage 2
Lane 4 = sample collected at stage 4
Lane 6 = sample collected at stage 6

Figure 5 PCR amplification of *lectin* (a), CaMV 35S promoter (b), *nos* terminator (c) and *EPSPS* (d) from genomic DNA of the samples collected at each step of experimentally tofu making

Table 3 The *EPSPS* content (%) contained in each sample of tofu products

Sample of tofu products collected at each stage	*EPSPS* (%)*
1, (raw soy grain)	5.294
2, (the so-call "go")	5.544
3, (so-call "okara)	5.438
4, (liquid soymilk)	5.887
5, (boiled-liquid soymilk)	4.572
6, tofu (momen-tufo)	5.910

*Values are the mean of four replicates with standard deviation (SD) = 0.15.
The average means are not significantly different using *F*-test ($P< 0.05$).

Although our results indicate that the transgene, i.e., *EPSPS* was not degraded by food processing and only 2.8% of local tofu were contaminated with GM material. Using the data presented by Jonas *et al*. [8], the ratio of recombinant DNA per soy genome in commercial GM soybean was about 0.00018. By combining these two figures, chance of consumer exposed to transgenes was very small. This implied that contamination of *EPSPS* in tofu was still in the safe level and well below the 5% level allowed by the Thai labeling policy.

4. CONCLUSIONS

Our results indicated that 3 out of 106 samples (ca. 2.8%) were contaminated with CaMV 35S promoter, *nos* terminator DNA fragments and *EPSPS*. Processing steps such as grinding and boiling had no effect on degradation of *EPSPS* during the process of tofu making. The amount of *EPSPS* was not different between GM raw soybean as starting materials and by-products derived from each processing step.

5. ACKNOWLEDGEMENTS

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