Qualitative and Quantitative Detection of GM Soy Grain, Soy Meal and Food Products

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ABSTRACT

This research was aimed to study the status and distribution of GM soy grain, meal and food products derived from GM soybean and maize. Samplings were made by collecting soy grain and meal from plant quarantines, the Department of Agriculture, some local grocery stores and food products from private firms, supermarkets and local grocery stores in Bangkok and vicinity areas around Bangkok during January 2002 – September 2005. Samples were then initially screened for GM grain, meal and food products by checking for the presence of 123 bp and 180 bp DNA fragements of CaMV 35S promoter and nos terminator respectively by using PCR. The positive samples were quantitatively analysed for 5-enol-pyruvyl-shikimate-3-phosphate synthase (*EPSPS*) by real-time PCR. Results showed that the ratio of GM soy grain and meal and food products were 5.2, 29.8 and 8.0% respectively. Trends in the distribution of GM soy grain, meal and food products showed different patterns during the years 2002-2005. In 2004, the ratio of GM soy grain, meal and food products sharply decreased. When the positive samples quantitatively analysed for *EPSPS*, the average percentage of *EPSPS* contaminated in GM soy grain was the highest at 94.2%, while the contaminated level of GM soybean food products was at 3.7% which was not higher than the Thai standard level (5%).

Key words: qualitative and quantitative analysis, soybean, food products, PCR, real-time

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Introduction

Hundreds of GM plants mainly soybean, maize and cotton have been authorized for commercialization in many countries since the first genetically modified (GM) tomato known as FlavrSavr was approved for commercialization in 1994. The GM soybean is one of the top two most cultivated GM plant (Shehata, 2005). The inserted elements mostly found in GM soybean consists of the 35S promoter (CaMV 35S) and the nopalin synthase terminator (*nos*), which are derived from the cauliflower mosaic virus and the bacteria, *Agrobacterium tumefaciens* respectively, and the CP4 *EPSPS* of *Agrobacterium tumefacien* strain CP4, which confers herbicide tolerance especially to glyphosate (Anklam *et al.*, 2002).

Soybean is one of the major crops used as raw materials for a wide variety of food products. It is also used in food and feed industries in Thailand. The use of soybean and its products in Thailand continuously increases every year. GM soybean is not allowed to be commercially cultivated in Thailand. However due to insufficient domestic grain production, soybean grain is also imported into Thailand, as well as processed food or food products derived from GM soybean, but that from such imports, GM soybean may potentially appear in Thai markets and contaminate into food chain. Contamination of GM soybean in the Thai food chain has raised a number of questions such as food safety and environmental risk. Although there is no direct evidence that the three inserted elements of GM soybean have any harmful effect on the human health, contamination of GM soybeans into the food chain still causes considerable anxiety to consumers. This subject has become one of the main issues of public discussion.

Thailand is an open country and agrees on free trade and as such it is impossible to obstruct the import of GM soybean grain and food into the country. Although, the Thailand authorities have released several strategies to regulate GM plants and food, these strategies seem to be insufficient to relieve public anxiety. Supplemental strategies are thus required. Therefore, regular detection of GM soybean and food products derived from GM soybean and reporting the status to the public is one essential new strategy. The aim of this research was to study the status and distribution of GM soy grain, meal and food products derived from GM soybean in Thailand.

Materials and Methods

1. Raw soy grain, soy meal and food samples

Some of raw soy grain and food products were collected and gathered randomly from private firms, local supermarkets and grocery stores mainly in Bangkok and vicinity areas around Bangkok. Some raw soy grain and meal were occasionally obtained from plant quarantines, the Department of Agriculture.

2. Reference standards

Each certified reference materials (CRM) standards was consisted of dried soybean powder with 0, 0.1, 0.5, 1, 2 and 5% (w/w) Roundup Ready™ soybean (RRS, IRMM 410R) and dried maize flour with 0, 0.1, 0.5 and 2% (w/w) Bt-176 maize (IRMM 411), respectively. The strandard was used as positive and negative controls for the detection analysis, produced by the Institute for Reference Materials and Measurements (IRMM) and commercialized by Fluka.

The procedure for qualitative and quantitative analysis of GM soy grain, meal and food product was diagrammatically outlined (Figure 1).

3. Sampling and preparation of raw soy grain, soy meal and food products

A total of 315 raw soy grain and soy meal and 138 food products were randomly obtained from a different sources as described in the section 1.

One kilogram of each sample raw soy grain and soy meal was ground and coded for further analysis.

One hundred and thirty eight food products including a variety of processing steps and types from relatively mild treated soybeans and ground maize to highly processed products containing soy and/or maize ingredients, were divided into 4 groups namly 1) food products derived from soybean, 2) other food products, 3) seasoning powder and 4) animal feed for further analysis. One kilogram of each sample food products in solid form was ground and coded prior to DNA extraction. In the case of food products in liquid form, 1,000 ml of each sample was centrifuged, the precipitate collected, mixed well and coded for further assay.

All raw soy grain, soy meal and food samples were randomly collected durring January 2002 – September 2005.

4. DNA extraction and purification

A different DNA extraction method

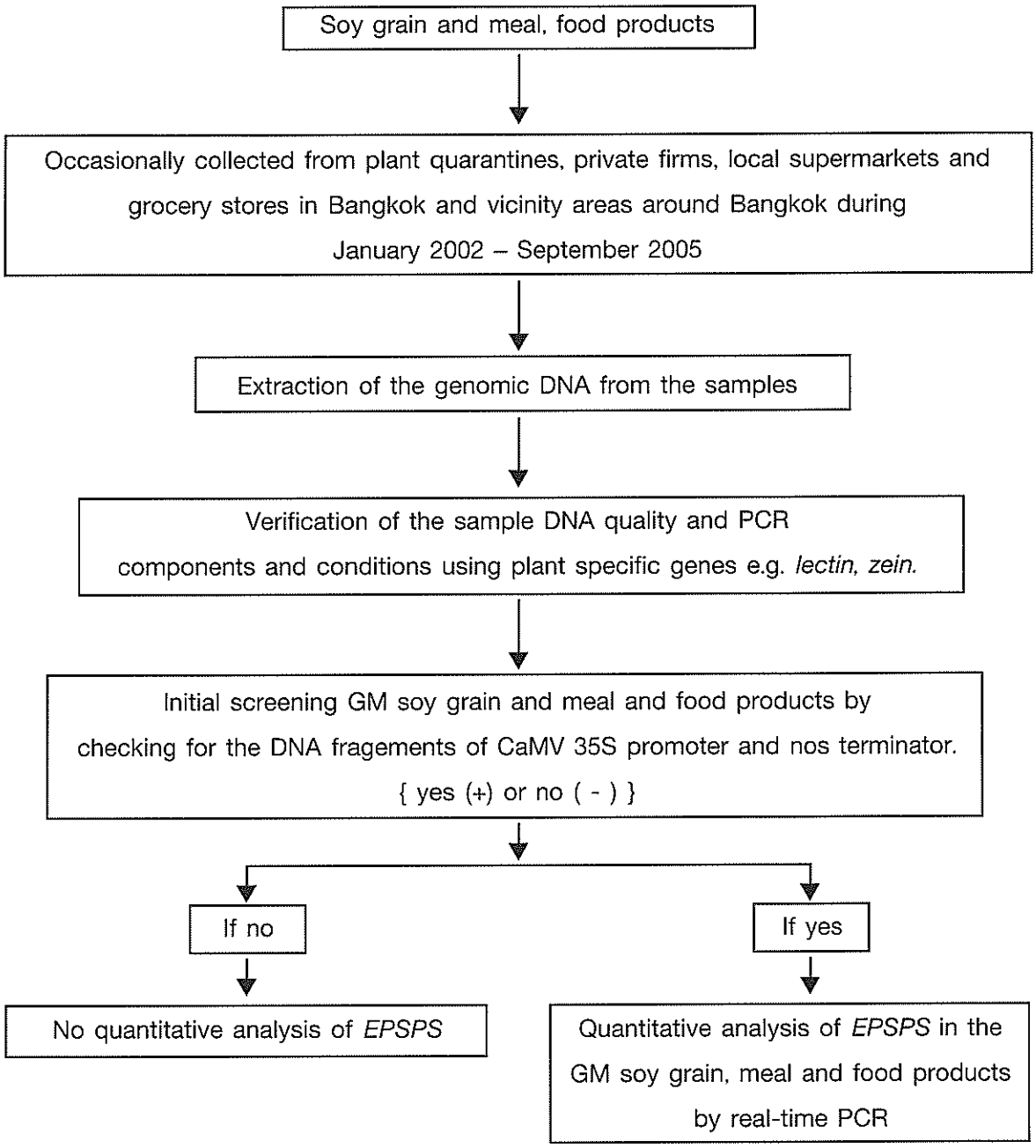

Figure 1. Schematic procedure of analysis

was applied to the samples which were depending on the types of samples. The raw soy grain, soy meal, and solid food products derived from soybean, animal feed and other food products, each ground raw soy grain, soy meal sample (0.5 g) and ground food products sample (2.0 g), genomic DNA were extracted by the guanidinium-chloroform method (Studer,1997). The food products in liquid form seasoning liquid at 2.0 ml of each sample were subjected to DNA extraction using

GeneScan DNA extraction kit (Eurofins GeneScan). Genomic DNA of each sample was then purified using Wizard® Miniprep DNA Purification Kit (Promega., USA) and DNA concentration was spectophoto metrically adjusted to 50 ng/μl.

5. Qualitative PCR screening of GM raw soy grain, soy meal and food products

Prior to screening of GM raw soy grain, soy meal and food products, the quality of the extracted genomic DNA of each sample and the PCR conditions were examined by amplification of the DNA fragments of the soybean lectin and maize zein gene using the primers Lec1/ Lec2 and Zein-F/ Zein-R (specific to the soybean lectin and the maize zein genes, respectively (Table 1). Samples of food products containing both soy and maize ingredients were also examined with these primers. Screening of the GM raw soy grain, soy meal and food products was then verified by the primers 35SF2/ 35SR2 and Nos1/ Nos2 for the amplification of CaMV 35S promoter and nos-terminator respectively (Table 1).

All PCR was carried out in a reaction mixture containing 5.0 μl of 10x PCR buffer, 1.0 μl of 10 mM dNTP, 3 μl of 25 mM MgCl2, 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 strile DI water were also used as positive and negative control 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 (Table 1). The PCR products were analyzed by electrophoresis on a 1.0% (w/v) agarose gel.

6. Quantitative analysis of GM raw soy grain, soy meal and food products by real-time PCR

6.1 Construction of a standard curve

The reference standard genomic DNA was extracted from 5% (w/v) Roundup Ready dried soybean powder (5% RRS) according to protocol using guanidinium-

Table 1. List of PCR primers used in this study

Target DNA fragment	Primers	Primer sequence	Annealing T (°C)	Amplicon length (bp)
Lectin	Lec 1	5'-GACGCTATTGTGAGCTCCTC-3'	57°	181
	Lec 2	5'-TGTCAGGGCCATAGAAGGTG-3'		
Zein	Zein-F	5'-CCTATAGCTTCGCTTCTTCC-3'	56°	158
	Zein-R	5'-TGCTCTAATAGCGCTGATGA-3'		
CaMV 35S	35SF2	5'-GCTCCTACAAATGCCATCA-3'	60°	195
promoter	35SR2	5'-GATAGTGGGATTGTGCGTCA-3'		
Nos	Nos 1	5'-GATAGTGGGATTGTGCGTCA-3'	54°	180
terminator	Nos 2	5'-TTATCGTAGTTTGCGCGCTA-3'		

chloroform (Studer,1997) and purified using Wizard® Miniprep DNA Purification Kit (Promega, USA). The reference standard genomic DNA was then serially diluted to 1/4, 1/16 and 1/64 times with sterized pure DI water, respectively. Each RRS content of reference standard genomic DNA was spectophotometrically adjusted to 50 ng/μl and used as DNA template in real-time PCR for standard curve construction.

A standard curve based on threshold cycles (C_t) for 4-fold dilution series of the RRS reference standard genomic DNA was constructed for the GM soybean. C_t values were calculated by the Lightcycler™ software program version 3.5.28 (Roche Diagnostics, Thailand) to indicate significant fluorescence signals rising above background during the early cycles of the exponentially growing phase of the PCR amplification process. A standard curve was obtained by plotting the C_t value, which was defined by the crossing cycle number or crossing point, versus the logarithm of the concentration of each 4-fold dilution series of RRS reference standard genomic DNA (Figures 2 and 3). To verify the accuracy of the standard curve, 1% RRS reference standard genomic DNA was also parallely run with the four dilution series (Figure 3a).

6.2 Real-time PCR amplification

The genomic DNA of positive samples in the section 2.5 was quantitatively analyzed for the lectin and *EPSPS*, respectively and used as the DNA template in real-time PCR amplification using the

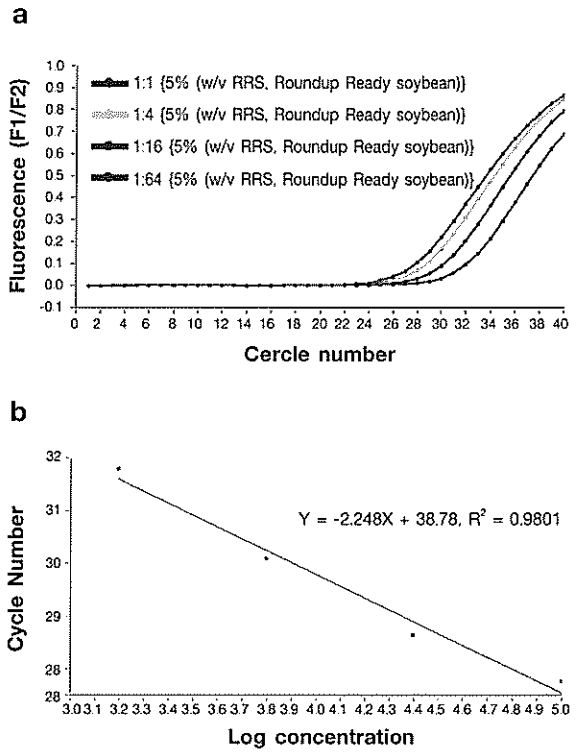

Figure 2. Real-time PCR amplification of *lectin* with serially diluted RRS reference standard genomic DNA as template: **a,** kinetics of fluorescence signal at different concentrations of standard genomic DNA using Sltm1 and 2 primers and the Sltmp probe (Table 2), and **b,** standard curve obtained by plotting the log concentration of DNA versus the cycle number.

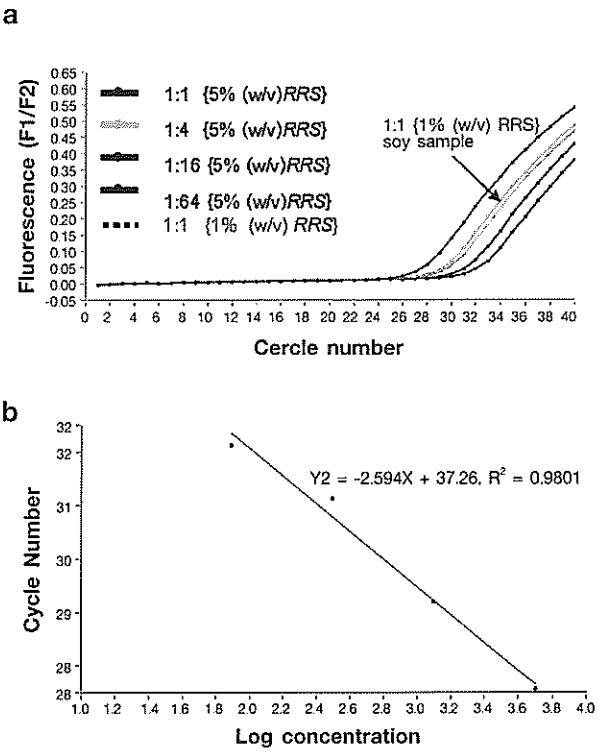

Figure 3. Real-time PCR amplification of CP4 EPSPS with serially diluted RRS reference standard genomic DNA as template: **a,** kinetics of fluorescence signal at different standard genomic DNA using Sttm 2a and 3a primers and the Sttmpa probe (Table 2), **b,** standard curve obtained by plotting the log concentration of DNA versus the cycle number.

primers and probes (Table 2). Real-time PCR amplifications were carried out in glass capillaries (Roche Diagnostics) in a total volume of 20 μl using a Lightcycler™ (Roche Diagnostics). Each reaction mixture contained 5.0 μl (10ng/μl) of each DNA template from positive samples, 10.0 μl of 2x quantitect probe PCR, 0.4 μl (5 pmol/μl) of each primer, 0.4 μl of TaqMan probe (1.5 pmol/μl) and 3.8 μl of sterile distilled water. Negative control reaction contained the same mixtures with 5.0 μl of sterile

Table 2. Sequence of primers and probes used in real-time PCR

Primer/probe	Specificity	Sequences
SItm1 primer		5'-AACCGGTAGCGTTGCGAG-3'
SItm2 primer	*Lectin*	5'-AGCCCATCTGCAAGGCTTT-3'
SItmp probe		5'FAM-TTCGCCGCTTCTTCAACTTCACCT-TAMRA-3'
Sttm3a primer		5'-GCAAATCCTGTAGCCTTTCC-3'
Sttm2a primer	CP4 *EPSPS*	5'-CTTGCCCGTGTTGATAACGTC-3'
Sttmpa probe		5' FAM-TTCATGTTCGCGGTCTCGCG-TAMRA-3'

distilled water replacing the DNA template. Real-time PCR amplification was performed according to the following real-time PCR step-cycle program : initial denaturation at 95°C for 90 s. followed by 40 cycles of amplification at 95°C for 5 s and at 60°C for 60 s and finally cooling at 40°C for 30 s.

6.3 Determination of *EPSPS* content

Using each standard curve, the number of lectin (Y1) and CP4 *EPSPS* (Y2) copy genes were calculated by substituting log concentration value of the DNA samples in each linear regression equation (Figures 2b and 3b). The GMO percentage in each positive sample was determined as being the ratio of the CP4 *EPSPS* copy gene number to the *lectin* copy gene number multiplied by 100.

Results and Discussion

1. Amplification of target DNA fragments

The amplificability of the DNA extracted from the samples, positive and negative controls were confirmed using plant-specific primers for *lectin* and *zein*. These results indicated that quality of extracted DNA and the PCR condition were reliable for further analysis of amplicon of this size (Figure 4).

2. Screening of GM samples

Most of the available GMOs contain any of the three genetic elements: the cauliflower mosaic virus (CaMV) 35S promoter, the nopalin synthase (nos) terminator or the kanamycin-resistance marker genes (nptII) (Ahmed, 2002). From January 2002 – September 2005 , 191, 124 and 138 samples of raw soy grain, soy meal and food products respectively, were screened for the presence of the two transgenic material in the samples; the

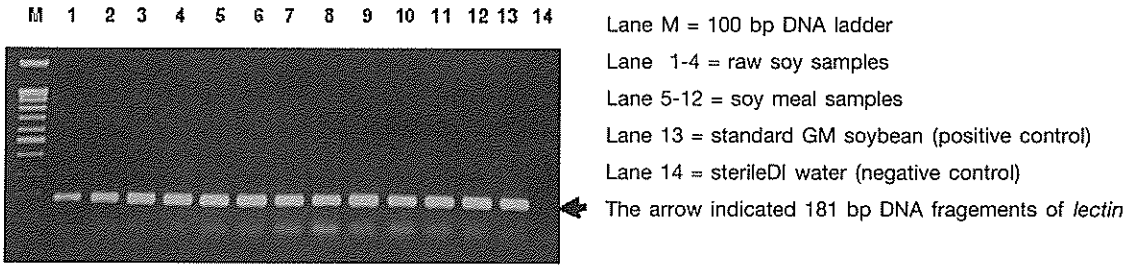

Figure 4. Amplification of 181 bp DNA fragements of *lectin* from some soy genomic DNA samples using Lec 1 and 2 primers

CaMV 35S promoter and the nos terminator (Table 3).

Results showed that 10 out of 191 (5.2%), 37 out of 124 (29.8%) and 11 out of 138 (8.0%) samples of raw soy grain, soy meal and food products respectively, were positive for the two introduced genetic elements (Table 3). The number of GM samples in each group ploted aganist the year revealed that the distribution pattern of each group was different during this time period but all tended to decrease in the third year (Figure 5). Decrease of GM samples in each group in Thai market could be influenced by the international regulation of labeling and traceability of GMOs and food and feed products produced from GMOs (Shehata, 2005). The percentage of GM soy meal was higher than those of GM raw soy grain and food products. This was likely due to that GM plants, animals and other organisms are not allowed to be commercially cultivated in Thailand and GM food products must first be approved for safe use according to Thailand's regulations. Therefore, a total of 315 raw soy grain and soy meal randomly sampled, 169 samples (53.7%) was imported into Thailand as raw materials for the animal feed industry. Of the 315 raw soy grain and soy meal samples, the number of positive samples per the number of the samples analyzed was classified according to their derived sources as shown in Figure 6. Our survey showed that the highest ratio of GM raw soy grain and soy meal was derived from the GM soybean producing countries as reported by James (2002).

As a total of 138 samples of food products were screened from which only 11 positive GM samples were found (Table 3). All of the GM positive food products were

Table 3. The number of samples collected and the number of positive samples contaminated with the CaMV 35S promoter and the nos terminator between year 2002 and 2005.

Sample type	The number of samples collected (a) and the number of GM samples detected (b) in each year								Total numbers of		GMOs (%)
	2002		2003		2004		2005		(a1)+(a2)+ (a3)+(a4)	(b1)+(b2)+ (b3)+(b4)	
	(a1)	(b1)	(a2)	(b2)	(a3)	(b3)	(a4)	(b4)			
Raw soy grain	99	0	17	4	54	6	21	0	191	10	5.2
Soy meal	4	3	36	25	83	9	1	0	124	37	29.8
Food products	25	7	53	2	25	1	35	1	138	11	8.0

derived from soybean. Five were animal feed and 3 of each were soy-flour and protein. No amplification of the DNA fragments of the CaMV 35S promoter and the nos terminator was detected for seasoning liquid and powder and other food products such as soy sauce, soya milk, pepper powder, maize soup and rice crackers. It is reasonable to expect that some of these samples were not made from GM plants.

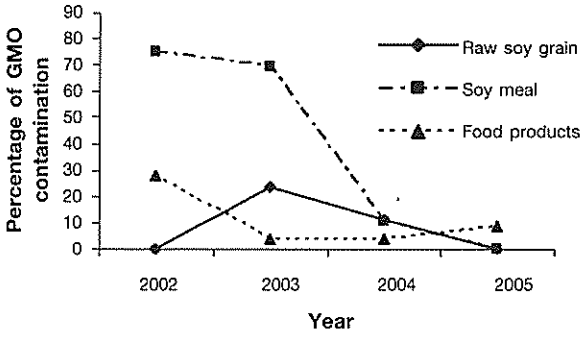

Figure 5. Distribution of GM raw soy grain, soy meal and food products during year 2002-2005

3. Quantitative analysis of GM raw soy grain, soy meal and food products by real-time PCR

Samples of raw soy grain, soy meal and food products showing positive PCR amplicons for the CaMV 35S promoter and the nos terminator were quantitatively analyzed for the *EPSPS* of *Agrobacterium* sp. strain CP4. The percentage of *EPSPS* contaminated in those samples were derived from soybean (Table 4).

In considering the percentage of EPSPS contamination in the average percentage of *EPSPS* contamination in raw soy grain was 27.6 and 90.5% higher than those of the soy meal and food products respectively (Table 4). This result was in accordance with that reported by Jonas *et*

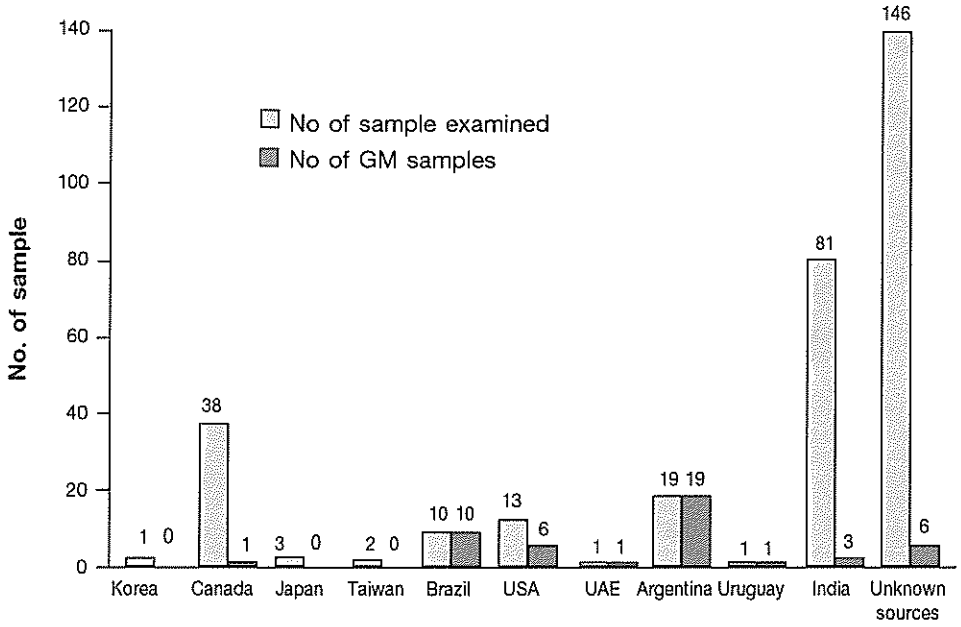

Figure 6. The number of samples of raw soy grain and soy meal examined and the number of GM samples detected in imports from the indicated countries.

Table 4. Quantification of *EPSPS* contaminated in each type of samples by real-time PCR

Type of samples	Quantity of *EPSPS* (%)[1/]
Raw soy grain	94.2 ± 4.5 a[2/]
Soy meal	66.6 ± 6.8 b
Food products	3.7 ± 2.4 c

[1/] Values are the mean of ten, thirty seven and eleven replicates + SEM for raw soy grain, soy meal and food products respectively. Ten thirty seven and eleven replicates of each positive sample types were from (Table 3).

[2/] Values in the same column followed by a common letter are not significantly different at 5% level by Dunnett's.

al. (2001) who suggested that the higher appearent transgene level in raw grain than in processed food was because the processing treatments such as grinding and heat treatment could degrade or breakdown the DNA into small fragments. Likewise, difficulty in detecting Roundup Ready soybean in some highly processed products was also confirmed by Zhang *et al.* (2007). In this study, the average percentage of *EPSPS* contamination in food products reached 3.7% but this figure includes animal feeds as well (the average percentage of *EPSPS* in animal feeds ≈ 7.08 %). If animal feed is

excluded, the actual average percentage of *EPSPS* contamination in food products derived from soybean was only 0.8%, well below the 5% level allowed by the Thai labeling policy.

Conclusion

The four year survey of genetically modified soybean and food products in Thai markets revealed that there were GM raw soybean, soy meal and food products made from GM soybeans. The distribution pattern among GM raw soybean, soy meal and soy-made food products was different and not consistent but all revealed a decrease by the 3rd year. About 53.7% of GM raw soybean and soy meal were imported from overseas. The percentage of *EPSPS* contamination in soy-made food products excluding animal feed was only 0.8% which was still in the safe level. However, it should be kept in mind that this survey may not accurately or thoroughtly reflect the real picture of GM soybean and derived food products in Thai markets because of the limited sampling method. Both sample sizes and frequency of sampling can affect the results obtained.

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