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Development of multiplex-PCR for Genetically Modified Organism (GMO) detection targeting EPSPS and Cry1Ab genes in soy and maize samples

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Abstract: The incidence of GMO is increasing worldwide therefore development of a reliable yet cost and time saving analytical method to detect GMO is important. This study aimed to develop a multiplex-PCR for GMO detection targeting Cry1Ab and EPSPS genes in soy and maize samples simultaneously, and secondly to obtain purified nucleic acids using CTAB DNA extraction method for conducting a GM specific analysis on various types of food samples. The multiplex PCR was optimized to improve PCR performance and to minimize failure. Out of 60 samples, 42 (70.0%) were found containing Cry1Ab or EPSPS genes, consisting of 11.9% of Roundup Ready Soya positive samples and 88.1% of Bt 176 Maize positive samples. Besides, 71.7% samples yielded DNA concentration above 50 ng/µl; 66.7% samples were in the DNA purity range of 1.6 to 2.0 and 85.0% of the samples were amplifiable for the endogenous gene screening. The CTAB DNA extraction method is effective for the DNA extraction from animal feeds, raw materials and processed foods.

Keywords: Multiplex-PCR, Genetically Modified Organism (GMO), Cry1Ab, EPSPS

Introduction

Genetically Modified Organism (GMO) can be defined as organisms in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating or natural recombination, for instance by being genetically modified (GM) or by recombinant DNA technology (Anklam *et al*., 2002). GM food as defined by the European Community (EC) Novel Food Regulation, is a food which is, of which is made from a GMO and contains genetic material of protein resulting from the modification. Some common examples of GMO include herbicide tolerant soybean, insect pest resistant maize, virus resistant papaya and salt resistant tomato.

The use of genetically modified organisms in food products of as food is getting more widespread over the years. A huge variety of food crops have been genetically modified to contain beneficial traits for example herbicide tolerance, insect or pest resistance and disease tolerance. The two most cultivated GMOs are maize and soya, which represent the staple constituents of many foods (Meyer *et al*., 1999).

In this study, the two genes involved are 5-enolphyruvylshikimate-3-phosphate gene synthase (EPSPS) and Cry1Ab genes. The presence of EPSPS gene identifies the Roundup Ready event in soybean products. According to AGBIOS, Roundup Ready Soya contains a glyphosate tolerant form of the plant enzyme 5-enolphyruvylshikimate-3-phosphate gene synthase (EPSPS) isolated from the common soil bacterium, Agrobacterium tumafaciens strain CP4 (CP4 EPSPS).

On the other hand, the presence of Cry1Ab gene confirms the Bt 176 event in maize products. The Bt-176 maize was developed to be resistant to attack by European corn borer (*Ostrinia nubilalis*), which is a major insect pest of maize in many countries and proven difficult to handle by conventional approach. Currently, the Bt-maize Event 176 is subjected to European Commission's decision on withdrawal from the market.

GMO detection plays a vital part in food safety because it assists the labeling process of GM products. Labeling does not act as a safety guideline or make the food acceptable, but it provides sufficient information for consumers to make an informed choice. Pressure from consumer groups and public demand has led several countries to require labeling for the presence of GMOs in foods (Matsuoka *et al*., 2002).

Different countries set the GMO labeling threshold based on their own criteria. To date, European Union (EU) is still the one and only that sets the GMO threshold level of 0.9%, which is most

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stringent legislation globally (Heide *et al*., 2008). Australia, New Zealand, Brazil and Saudi Arabia have set GM food labeling threshold of 1.0%, while for South Korea it is 3.0%. (Viljoen *et al*., 2006). Japan and Taiwan set a 5.0% GM food labeling threshold level. In Malaysia, the Biosafety Bill was approved by Parliament in July 2007, allowing regulations pertaining to labeling of GMOs in food and feed to be introduced and enforced in the near future (Jasbeer *et al*., 2008). A 3.0% GMO labeling threshold has been approved but is yet to be implemented.

For this reason, a reliable and rapid multiplex-PCR is essential for the identification of GM materials in raw and processed food as well as in animal feeds. Because of unparalleled sensitivity and specificity, polymerase chain reaction (PCR) in its various formats, is currently the leading analytical technology employed in the qualitative and quantitative analysis of GMOs (Anklam *et al*., 2002). Prior to PCR analysis, DNA from the samples must be isolated. Though the CTAB DNA extraction is time consuming, it is still the method of choice in many studies because it produces low degraded and amplificable DNA from most food samples (Mafra *et al*. 2008). Previous work as reported by Cheah *et al*. (2006) indicated Roundp Ready Soya can be semi-quantitated with the combined approach of PCR and membrane based technique.

With the development of a rapid multiplex-PCR, screening and identification of GM materials in samples can be carried out rapidly and at a cost-saving way. This approach will assists in the GMO labeling process as positive GM samples can be discriminated easily and subjected for further quantitative analysis.

Therefore, this study was conducted to develop a Multiplex-PCR targeting Cry1Ab and EPSPS genes for the identification of GM materials in maize and soy samples simultaneously, as well as to obtain purified nucleic acids using CTAB DNA extraction method for conducting a GM specific analysis on various types of food samples.

Materials and Methods

Sample collection

A total of 60 samples consisting of 10 animal feeds, 17 raw materials and 33 processed foods containing soybean or maize or both were collected randomly from the local retailed markets and supermarkets in Selangor, Malaysia. A few of the samples were taken from Animal House of Faculty of Medicine and Health Science, UPM. In this study, Roundup Ready GM-soybean powder with 5.0% GMO content and Bt 176 maize powder with 2.0% GMO content were used as positive controls. These certified reference materials (CRMs) were developed by the Institute for Reference Materials and Measurement (IRMM, Geel, Belgium) and stored at -20°C until use.

DNA extraction and quantification

Cetyltrimethylammonium bromide (CTAB) method was utilized to extract DNA from all samples. All solid samples were grounded into fine powdery form prior subjecting to extraction procedure. A total of 200 mg sample was mixed with 300 µl of ultrapure water and 700 µl of CTAB buffer (20 g/l CTAB, 1.4 M NaCl, 0.1 M Tris-HCl, 20mM Na2EDTA). 10 µl of RNase (10 mg/ml) and was added and each incubated at 65°C for 30 min, followed by addition of 10µl Proteinase K (20 mg/ml) and incubated at 65°C for 30 min. The mixture was centrifuged at 12,000 g for 10 min, then washed twice with 500 µl chloroform, centrifuged at 12,000 g for 15 min and 5 min each until phase separation. The supernatant was transferred and added with 2 vol of CTAB-precipitation solution (5 g/l CTAB, 0.04 M NaCl) and incubated at room temperature for 60 min then centrifuged at 13,000 rpm for 5 min. The precipitate was dissolved in 350 µl of 1.2 M NaCl and 350 µl chloroform then added with 0.6 vol of isopropanol after centrifugation at 12,000 g for 10 min, followed by incubation for 20 min at room temperature. After another round of centrifugation at 12,000 g for 10 min, the supernatant was discarded and 500 µl of 70% ethanol was added and centrifuged at 12,000 g for 10 min. Finally the supernatant was discarded and pellet was dried, followed by dissolving the DNA in 50 µl or 100 µl of sterile TE buffer (Tris/HCl 10 mM, EDTA 1 mM, pH 7.0), depending on the size of pellet. The DNA was finally stored in -20ºC until use.

The quality and quantity of extracted DNA was analyzed using Eppendorf Biophotometer. A ratio of absorbance at 260 nm and absorbance at 280 nm (A260/A280) was used to determine the purity of DNA. The program was set up for double stranded DNA. 1.0 µl of DNA template was mixed with 49.0 µl of ultrapure water in the cuvette then subjected for quantification.

Endogenous gene screening

Three endogenous genes were used to screen for presence of housekeeping genes in soy or maize in the samples. Primers Lec1 (GTG CTA CTG ACC AGC AAG GCA AAC TCA GCG) / Lec2 (GAG GGT TTT GGG GTG CCG TTT TCG TCA AC) were used to amplify lectin gene in soy samples with amplicon size of 164 bp (Vollenhofer *et al*., 1999);

whereas for maize samples, two primers pairs were used. Inv-F (CCG CTG TAT CAC AAG GGC TGG TAC C) / Inv-R (GGA GCC CGT GTA GAG CAT GAC GAT C) was used to screen for invertase gene with amplicon size of 226 bp (Ehlers *et al.*, 1997); while Ze03 (AGT GCG ACC CAT ATT CCA G) / Ze04 (GAC ATT GTG GCA TCA TCA TTT) was used to screen for zein gene with amplicon size of 277bp (Pauli *et al*., 2000). All the oligonucleotide primers were diluted to working concentration of 10 pmol/µl with ultrapure water and stored at -20ºC until use.

Three different singleplex PCRs were performed using Eppendorf Thermocycler (Germany) for amplification of endogenous genes for soy and maize samples.

For *lectin* gene screening in soy samples, the final volume of each reaction was 20.0 µl that comprised of 11.7µl of ultrapure water, 2.0 µl of 10x i-*Taq*™ PCR buffer (350 mM HCl (pH 9.0), 250mM KCl, 35 mM $MgCl_2$ and enhancer solution) (iNtRON Biotechnology, Korea), 2.0 µl of dNTPs, 1.0 µl of each forward and reverse primers, 0.3 µl of 5U/µl i-*Taq*™ polymerase and finally 2.0 µl of DNA template. Conditions of amplification used were as follows: predenaturation at 94ºC for 3 min, followed by 40 cycles of 1 min at 94ºC, 1 min at 58ºC and 2 min at 67ºC, then a final extension at 68ºC for 7 min.

For *invertase* gene screening in maize samples, the final volume of each reaction was 20.0 µl that comprised of 13.25 µl of ultrapure water, 2.0 µl of 10x i-*Taq*™ PCR buffer, 1.5 µl dNTPs, 0.5 µl of each forward and reverse primers, 1.0 µl of DMSO, 0.25 µl of 5U/µl i-*Taq*™ polymerase and finally 1.0 µl of DNA template. Conditions of amplification used were as follows: predenaturation at 95ºC for 8 min, followed by 40 cycles of 30 s at 95ºC, 35 s at 60ºC and 35 s at 72ºC, then a final extension at 72ºC for 7 min.

For *zein* gene screening in maize samples, the final volume of each reaction was 20.0 µl that comprised of 12.7 µl of ultrapure water, 2.0 µl of 10x i-*Taq*™ PCR buffer, 2.0 µl dNTPs, 1.0 µl of each forward and reverse primers, 0.3 µl of 5U/µl i-*Taq*™ polymerase and finally 1.0 µl of DNA template. Conditions of amplification used were the same as for *lectin* gene screening.

Multiplex PCR amplification

The primer pair used to screened for EPSPS gene was RR01 (TGG CGC CCA AAG CTT GCA TGG C) / RR04 (CCC CAA GTT CCT AAA TCT TCA AGT) with amplicon size of 356 bp (Abdullah *et al*., 2005); whereas for Cry1Ab gene, Cry1Ab (ACC ATC AAC AGC CGC TAC AAC GAC C) /Cry1As (TGG GGA ACA GGC TCA CGA TGT CCA G) primer pair with amplicon size of 184 bp was used (Tengel *et al*., 2001). The oligonucleotide primers were diluted to working concentration of 10 pmol/µl with ultrapure water and stored at -20ºC until use.

The hotstart multiplex- PCR for amplification of EPSPS gene and Cry1Ab gene was conducted using Eppendorf Thermocycler (Germany) with a final volume 20.0 µl comprised of 8.0 µl of ultrapure water, 2.0 µl of 10x PCR buffer (Finnzymes, Finland), 1.0 µl of DMSO, 2.0 µl of DNA template, 2.0 µl dNTPs, 1.0 µl of each forward and reverse the two primer pairs and 1.0 µl of 2U/µl Taq polymerase (Finnzymes, Finland). Conditions of amplification used were as follows: predenaturation at 95ºC for 8 min, followed by 40 cycles of 30 s at 95ºC, 35 s at 60ºC and 35 s at 72ºC, then a final extension at 72ºC for 7 min.

Agarose gel electrophoresis

Agarose gel was made visible under UV transilluminator Alpha Imager™ 2200 (Alpha Innotech, USA) after staining with ethidium bromide (0.5 µg/ml). A 100 bp DNA ladder (Geneaid) was used as a marker to detect the presence of the targeted bands.

For singleplex PCR in endogenous genes screenings, the amplification products were analyzed using 1.8% agarose gel electrophoresis in 1x TBE buffer for 45 minutes at 70 volts. As for multiplex PCR in GM event identification, the amplification products were analyzed using 3.0% agarose gel electrophoresis in 1x TBE buffer for 1 hour 50 minutes at 70. A higher concentration of agarose gel was used to enhance the separation of bands as well as to increase the resolution.

Statistical analysis

The statistical analysis was conducted using SPSS 16.0. Pearson Correlation Coefficient Analysis was conducted to test for relationship between DNA concentration and DNA purity while Fisher's Exact Test was performed to check for association between DNA amplificability and DNA concentration, as well as between DNA amplificability and DNA purity. The basic significance level in both analyses were fixed at $P<0.05$. Therefore, a P value more than 0.05 was considered not significant.

Results

Evaluation of CTAB DNA extraction method

DNA yield

Results summarized in Table 1 indicated that the mean yield of 60 tested samples was found to be 232.03 ng/µl. Across different types of samples, animal feeds showed highest mean DNA yield of 517.60 ng/µl, followed by raw materials with mean DNA yield of 306.41ng/µl and processed foods with mean DNA yield of 107.18 ng/µl.

When investigated across different types of samples, none of the animal feeds had DNA yield in the range of 0-50 ng/µl and 50.0% had DNA yield more than 300 ng/µl. 52.9% of raw materials had DNA yield in the range of 101-300 ng/µl. In processed food, majority had DNA yield in the range of 0-50 ng/µl (45.5%) and 101-300 ng/µl (36.4%).

Table 1. Mean and standard deviation of DNA yield

Samples	No. of samples	DNA Purity	DNA Yield (ng/µl)
Animal Feeds	10	Mean purity ± SD 1.72 ± 0.061	[Mean] ± SD 17.60 ± 655.162
Raw Materials	17	Mean purity ± SD 1.58 ± 0.335	[Mean] ± SD 306.41 ± 240.583
Processed Foods	33	Mean purity± SD 1.86±0.544	[Mean] ± SD 107.18 ± 115.504
Overall	60	Mean purity ± SD 1.76 ± 0.455	[Mean] ± SD 232.03 ± 335.475

DNA purity

The mean purity of a total of 60 samples was 1.75. If looked across different types of samples, the mean DNA purity was 1.72 for animal feeds, 1.86 for processed foods and 1.58 for raw materials.

All animal feeds had DNA purity in the range of 1.6 to 2.0. For raw materials, 52.9% had DNA purity in the range of 1.6 to 2.0 whereas for processed food, majority (63.6%) had DNA purity in the range of 1.60 to 2.00.

Generally, there was no significant correlation between DNA concentration and DNA purity when tested with Pearson Correlation Coefficient Analysis, whether in overall, or when investigated across different types of samples (Table 2).

Table 2. Correlation Coefficient Analysis between DNA concentration and purity

Types of samples	Correlation coefficient, r	Significant value (P)	Conclusion
Animal Feeds	0.078	0.831	Insignificant
Raw Materials	-0.061	0.815	Insignificant
Processed Foods	-0.078	0.666	Insignificant
Overall	-0.109	0.409	Insignificant

P < 0.05 was considered significant.

DNA amplifiability

Three singleplex PCRs were performed with three different primer pairs to screen for maize or soy endogenous genes. All 60 samples were analyzed and the result showed 51 out of 60 samples contained *lectin*, *invertase* or *zein* gene. Of these samples, 6.7% contained only lectin gene, 36.7% contained either *invertase* or *zein* gene, 41.7% contained both soy-specific and maize-specific genes, whereas the remaining 15.0% contained none of the endogenous genes screened. The result showed as high as 85.0% of the samples were amplifiable. When looked across different types of samples, 100.0% of raw materials were amplificabled, followed by 80.0% animal feeds and 79.0% processed foods.

In Fisher's Exact Test, all P values were found to be above 0.05 therefore concluded there was no significant relationship between DNA yield and DNA amplificability, as well as between overall DNA purity and DNA amplificability (Table 3).

Table 3. Fisher's Exact Test on DNA concentration and DNA amplificability

Types of samples	Exact Signifance (2-sided) (P)	Conclusion
Overall	0.256	Insignificant
Processed Foods	0.674	Insignificant

P < 0.05 was considered significant

GM analysis

The GM analysis was performed with multiplex PCR analysis using two pairs of primers (RR01/RR04 and Cry1Ab/Cry1As) that amplified 356 bp fragment and 184 bp fragment respectively. The PCR products were easily resolved in 3.0 % agarose gel electrophoresis as shown in Figure 3.

Table 4 summarized that 42 (70.0%) out of 60 samples tested were positive for the GM events of this study, which are the Roundup Ready Soya event and the Bt 176 Maize event. Of these positive samples, 5 (11.9%) were found to contain EPSPS gene while 37 (88.1%) were found to contain Cry1Ab gene.

When investigated across different types of samples for Roundup Ready event, 4 (40.0%) animal feeds were found to contain EPSPS gene, followed by 1 (5.9%) in raw material and none (0.0%) in processed foods. As for Bt 176 Maize event, 3 (30.0%) animal feeds were found containing Cry1Ab gene, 12 (70.6%) in raw materials and 22 (66.7%) in processed foods.

From the two fold dilution, it was observed that in CRM (as in Figure 4), the limit of detection (LOD) was 7.6 ng/µl, whereas for samples (as in Figure 5), it was 6.2 ng/µl. Therefore the LOD of this multiplex system is 6.2 ng/µl.

Table 4. Outcome of GM screening across different types of samples and GM events

Types of Products	Number of samples	GM Event		Total
		Round Ready Soya	Bt 176 Maize	
Animal Feed	10	4	3	7
Raw Material	17	1	12	13
Processed Food	33	0	22	22
TOTAL	60	5	37	42

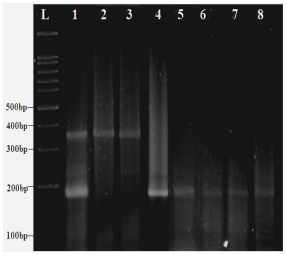

Figure 3. Representative agarose gel electrophoresis picture of PCR products of GM positive samples in GM screening, including animal feeds, raw materials and processed foods. Lanes: L, 100bp DNA Ladder; 1, Positive control; 2 & 3, Animal Feeds; 4, Raw material; 5, 6, 7 & 8, Processed Foods

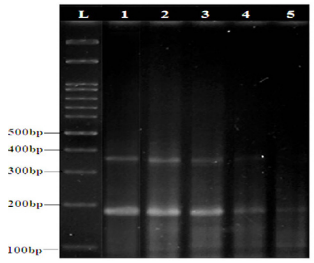

Figure 4. Agarose gel electrophoresis picture of PCR product from a two-fold serial dilution on equal mixture of CRM (5% RRS & 2% Bt 176) from IRMM. Lanes: L, 100bp DNA Ladder; 1, undiluted CRM 122.0ng/µl; 2, 3, 4 & 5, diluted CRM: 30.5ng/µl, 15.3ng/µl, 7.6ng/µl & 3.8ng/µl respectively

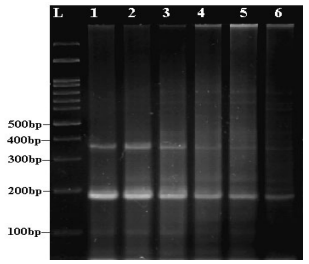

Figure 5. Agarose gel electrophoresis picture of PCR product from a two-fold serial dilution on equal combination of positive Roundup Ready Soy sample and positive Bt 176 maize sample. Lanes: L, 100bp DNA Ladder; 1, undiluted sample 198.0ng/µl; 2, 3, 4, 5 & 6, diluted samples: 99.0ng/µl, 49.5ng/µl, 24.8ng/µl, 12.4ng/µl & 6.2ng/µl respectively

Discussion

In this study, conventional CTAB DNA extraction method was used to isolate DNA from various types of samples including animal feeds, raw materials and processed foods. DNA extraction methods must be efficient, yielding as much DNA as possible from the sample matrix (Smith & Maxwell, 2007). The CTAB DNA extraction method can extract pure DNA of the high molecular weight from plants and is an efficient method for a wide range of plant materials and plant-derived foods by providing a good separation of DNA from polysaccharides (Jasbeer *et al*., 2008). According to Mafra *et al.* (2008), the extraction efficiency of the extraction protocol was evaluated by the determination of yield and purity of DNA extracts, as well as amplificability of DNA extracts. Therefore in this study, the CTAB extraction method was evaluated by looking from three different aspects, which are DNA yield, DNA purity and DNA amplification.

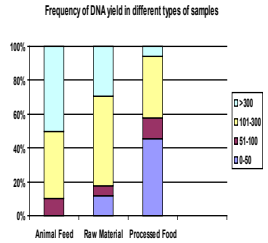

Figure 1. Bar chart showing frequency of DNA yield in different types of samples

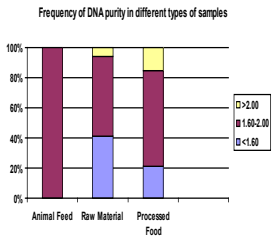

Figure 2. Bar chart showing frequency of DNA purity in different types of samples

Evaluation of CTAB DNA extraction method

DNA yield

When categorized into four different groups of DNA yield (<50 ng/µl, 51-100 ng/µl, 101 to 300 ng/µl and >300 ng/µl), 100.0% of the animal feeds had DNA yield more than 50 ng/µl, followed by 88.0% of raw materials (Figure 1). This was in agreement with a study conducted by Tung Nguyen *et al*. (2009), which had demonstrated that the CTAB method is applicable for extracting total DNA from animal feeds and raw materials, providing high DNA yield. As high as 45.5% of the processed food samples had DNA yield below 50 ng/µl. This finding is common in processed food due to degradation of DNA during food processing. This is in accordance with a study by Ahmed (2002) which revealed that heating and other processes associated with food production can degrade DNA. In addition, low DNA yield could be also due to the presence of PCR inhibitors (Margarit *et al*., 2006).

DNA purity

DNA purity can be affected by various types of contaminants in food matrices for example carbohydrates, peptides, phenols or aromatic compounds which can be reflected by absorption at 260 nm. A high-quality DNA extraction method should give not only good DNA yield but also good DNA purity (Tung Nguyen *et al*., 2009). Theoretically, an A_{260}/A_{280} ratio lower than 1.80 shows contamination with proteins and a ratio of more than 2.0 indicates contamination of RNA.

When categorized into three different groups of DNA purity (<1.6, 1.6 to 2.0 and >2.0), 100.0% of the animal feeds have DNA purity in the range of 1.6 to 2.0, followed by 63.6% of processed foods and 52.9% of raw materials (Figure 2).

With RNase treatment in the CTAB extraction protocol, there was still 11.7% of samples showing contamination of RNA (A_{260}/A_{280} more than 2.0). However, this did not affect the quality of DNA significantly. In this study, even a sample from category of processed food with DNA purity ratio as high as 2.73 was able to be amplified.

DNA amplifiability

For PCR, the target sequence within the DNA must be amplifiable. Amplification of DNA in PCR is influenced by the overall structural integrity of the DNA, as well as by the presence of co-purifying

inhibitors from the matrix or the extraction reagents, which can reduce the effciency of PCR (Smith & Maxwell, 2007).

The amplifiability of DNA extracted from samples were confirmed by PCR amplification using soybean specific (*lectin* gene) and maize-specific (*invertase* gene or *zein* gene) primers for samples containing soybean or maize respectively. As shown in the result section, 85.0% samples were amplifiable, indicating whether the samples contained either soybean or maize or both materials. The good amplifiable DNA of processed food was in agreement with a study which verified DNA extracted from highly processed food products using CTAB method demonstrated good amplification result (Mafra *et al*., 2008). Gryson *et al*. (2004) also demonstrated that even highly processed food extracted using CTAB extraction method could be amplified.

Presence of endogenous genes in the samples confirmed that the CTAB DNA extraction method used was adequate for the extraction of amplifiable soybean or maize DNA from the samples. For samples which were not amplified, either the DNA content was insufficient, or there were PCR inhibitors affecting them. Food samples comprise of complex mixture containing PCR inhibitors that may compromise the amplifiability of DNA (Pirondini *et al*., 2010).

Analysis on efficiency of CTAB extraction method

Overall, 71.7% of the samples have extracted DNA yield more than 50 ng/µl; 66.7% of the samples showed purity in the range of 1.6 to 2.0 and 85.0% of the samples were amplifiable for the endogenous gene screening. With these findings, we concluded that conventional CTAB DNA extraction method was effective to extract DNA from animal feeds, raw materials and processed foods. A coefficient correlation analysis done on DNA yield and DNA purity showed that there was no significant correlation between DNA yield and DNA purity ($r=-0.109$; $P = 0.411$), indicating DNA good in purity not necessary had good yield, and a DNA high in yield might not necessary have good purity, vice versa. Since there was no correlation in between DNA yield and DNA purity, Fisher's Exact Test was conducted to test for relationship between amplifiable DNA and DNA yield, as well as amplifiable DNA and DNA purity. The analysis revealed that the P values were 0.256 and 1.000 respectively; therefore concluded ability of DNA to be amplified was independent of the DNA yield and the DNA purity. This indicated it is still possible to amplify a sample eventhough the DNA yield and DNA purity may not be very good. PCR is very versatile and may tolerate slight contamination of DNA extracted using CTAB extraction method (Surzycki, 2000).

GM analysis

All over the world, regulations for the use and labeling of GM products are being implemented. Numerous analytical methods, including qualitative and quantitative, have been developed to determine the presence and/or the amount of GMOs in animal feeds, raw materials and processed foods.

Evidently, PCR is a highly reproducible and sensitive technique that can be successfully used in detecting transgenes for screening GM soybeans and GM maize (Rhandawa *et al*., 2006). As the number of GMO events on the market increases, it is clear that methods which can detect several targets in a single reaction, i.e. multiplex detection, is preferable when enforcing the legislation ruling the occurrence of GMOs in food (Heide *et al.*, 2008), whereby multiple targets are amplified in the same reaction simultaneously.

The main advantage of multiplex-PCR is that fewer reactions are needed to test a sample for potential presence of GMO-derived DNA (Forte *et al*., 2005). This detection method saves times and reduces costs (James *et al*., 2003). Therefore in this study, a multiplex PCR was optimized to detect EPSPS and Cry1Ab genes simultaneously.

In this study, after PCR amplifications of the *lectin*, *invertase* and *zein* genes, all samples were subjected to PCR amplification of EPSPS and Cry1Ab genes simultaneously using the developed hotstart multiplex PCR program. The two amplicons were 172 bp apart, making them appropriate to be used in the multiplex PCR as they can be separated well on agarose gel.

70.0% of the samples screened were found to be positive for the GM events of this study, which are the Roundup Ready Soya event and the Bt 176 Maize event. This finding was alarming because such high percentage of foodstuffs had been genetically modified.

The detection limit is defined as the minimum amount of DNA necessary to yield a visible signal on agarose gel after amplification (Gryson *et al*., 2004). This is also in accordance with Miraglia *et al.* (2004) which defined limit of detection as the lowest quantities that can be reliably detected. In this study, based on the two-fold serial dilution done on equal mixture of CRM (5% RRS and 2% Bt 176 from IRMM), as well as on equal mixture of positive RRS sample and positive Bt 176 sample, the limit of detection (LOD) of this multiplex PCR was found to be 6.2 ng/µl. The 6.2 ng/µl refered to the DNA

material and not the GM content.

Generally, the outcome of this analysis can assist the GMO labeling process by discriminating between positive and negative GM products. However, Further studies for the development of methods for detection of GMOs in processed food products, together with studies for the validation of analytical methods are necessary to ensure consumers' the safety and freedom of choice. (Forte *et al*., 2005). Therefore the positive GM results still require further quantitative analysis to detect the amount of GM content present. Quantification of GM content can be done by performing Real-Time PCR but it involves much higher cost.

Conclusion

Development of multiplex PCR assists the GMO labeling process to give consumers an informed choice. GMOs are still in their infancy in Malaysia, but GMO testing is needed as a precursor to supporting high standards of regulation, tracking development in GMO detection (Abdullah *et al.*, 2005).

Based on the findings in this study, it can be concluded that multiplex-PCR targeting Cry1Ab and EPSPS genes is able to identify GM materials in maize and soy samples simultaneously. This is evidenced by the results in this study which revealed the high prevalence of Bt176 maize and Roundup Ready soya in animal feeds, raw materials and processed foods in Malaysia. 70.0% of the samples screened were found to be positive for either one of the GM events.

Besides that, it is also concluded that the CTAB DNA extraction method is effective to extract DNA from various types of food samples because as high as 85.0% of the samples were positive for endogenous genes screening, proving the DNA extracted was of satisfactory yield and purity to be amplified.

In conclusion, with the increasing number of GM foods in the market, a reliable and rapid multiplex PCR is essential for the identification of GM material in soy or maize-containing animal feeds, raw materials as well as processed foods. Other than that, it is necessary to perform more and thorough studies in GMO quantitative analysis.

As for future direction, a duplex PCR is able to lead to development of tetraplex PCR by incorporating more transgenes, in order to detect more GM event in one reaction. Another possible aspect is the development of rapid GMO detection kit.

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