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Quantitative analysis of Roundup Ready soybean content in soy-derived food and animal feed by using Real-time PCR incorporated with cloned DNA fragments

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Abstract: Malaysia, Biosafety Bill 2006 was approved by Parliament in July 2007, and labeling legislation will be implemented soon. In this study, duplex polymerase chain reaction (PCR) was carried out to detect endogenous soybean *lectin* gene and exogenous *cp4-epsps* (5'-enolpyruvylshikimate-3-phospate synthase) gene simultaneously. Additionally, real-time PCR utilizing SYBR Green fluorescence dye were established for the quantitative analysis of Roundup Ready soybean (RRS), which is based on the two established calibration curve from cloned fragment of *cp4-epsps* gene and *lectin* gene respectively. Approximately, 39.5% (45/114) of the samples examined in this study contain RRS, animal feeds (31), processed food (13) and raw soybean (1). Additionally, 75.6% (34/45) of the positive samples were found contained RRS above 0.9%. The sensitive GMO quantitative approach described in this study enable the analysis of various samples and this will facilitate the labeling process.

Keywords: Genetically modified organisms, food and animal feed products, Roundup Ready soybean, PCR, cloned DNA standard, Quantitative Real-time PCR

Introduction

In the year 2008, the estimated global area planted with genetically modified (GM) crops reached 125 million hectares, and the main GM crop is soybean, which occupied the area of 65.8 million hectares (53% in global transgenic area) (James, 2008). Roundup ready soybean (RRS) from Monsanto is the world's most important GM crop (Berdal and Holst-Jensen, 2001). Due to rapid increase of GM crops in the market, and great public concern about the safety of food products and ingredient derived from genetically modified organisms (GMO), the government of some countries introduced mandatory-labeling legislation of GM food and their derivatives. The labeling threshold level was established in different countries, for example, 0.9% in the EU, 1% in Australia and New Zealand, 3% in Korea and 5% in Japan and Indonesia (Berdal *et al*., 2008). In Malaysia, mandatory labeling of food ingredients containing GM food will be implemented soon as the Biosafety Bill 2006 was approved by Parliament in July 2007 (Jasbeer *et al*., 2008). The information provided to the consumers through the labeling is important as an informed choice.

Practically, to maintain the cost-effectiveness of detection methods, qualitative duplex Polymerase Chain Reaction (PCR) method can be used in GMO screening on food products, and followed by, quantitative real-time PCR method for positive samples to obtain more precise numerical information for the labeling regulation (Di Pinto *et al*., 2008). To date, real-time PCR is considered most powerful tool in quantitative analysis of GMO due to its specificity and sensitivity (Shimizu *et al*., 2008). Other real-time approach on GMO detection includes suface plasmon resonance that enables real-time monitoring of molecule reactions via biospecific interaction analysis (Yoke-Kqueen and Son, 2010).

Real-time PCR utilizing SYBR Green I fluorescence dye that has high affinity for double-stranded DNA is the simplest and cost effective technique (Morrison *et al*., 1998). During PCR reaction, SYBR Green I fluorescence molecule will binds to the amplified PCR products, and thus produce fluorescence signal, which is then automatically pick-up and measured by the computer software. During the log-linear phase of amplification reaction, the emission of the fluorescence signal is proportional to the amount of specific PCR product (Terry and Harris, 2001). Many food products and ingredient that have been subject to physical and chemical processes, can lead to the size of the genomic DNA-fragments decreases. (Berdal *et al*., 2001). To overcome these problems, the suggested amplicon size is within the range of approximately 70-200 base pair(s) in order to

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obtain the highest achievable detectability (Berdal *et al*., 2001). Moreover, real-time PCR allows detection of low amounts DNA (Ahmad, 2000).

For GMO quantification analysis, the choice of reference materials or calibrators used to generate the standard curves is important. As previously reported, genomic DNA extracted from the certified reference materials (CRMs) from Institute of Reference Material and Measurement (IRMM) (Di Pinto *et al*., 2008) and cloned plasmid DNA fragments (Tavernier *et al*., 2004) have been used as calibrator.

The objectives of this study were to analyze the presents of RRS in the soy-derived food and feed products both qualitatively and quantitatively. Therefore, this study demonstrated the use of duplex PCR methods in screening of RRS for the commercially available soy-derived food products and animal feeds in Malaysia. In addition, the positive samples were subjected to quantitative analysis of RRS. Two calibration curves were generated with cloned fragments of exogenous *cp4-epsps* gene and endogenous *lectin* gene.

Materials and Methods

Samples

A total of 122 samples comprise of soybean-derived food products and animal feeds were randomly purchased from local supermarkets, traditional markets and grocery stores in Malaysia, as presented in Table 1. The textures of samples were categorized into solids and semi-solids. The certified reference material (CRM) was dried soybean powder, 5% genetically modified roundup ready soybean (RRS), developed by Institute for Reference Materials and Measurements (IRMM, Belgium) for the European interlaboratory trial. The CRM used in this study is similar as in the study reported by Yoke-Kqueen and Radu (2006). This 5% RRS was used as an external standard to validate the calibration curve. Beside, it can be used as a positive control to ensure the reproducibility and sensitivity of the both qualitative PCR and quantitative real-time PCR systems.

DNA extraction

A total of 100 mg/samples were ground into fine powder using a mortar and a pestle. However, semi-solid samples were ground under liquid nitrogen and subjected to DNA extraction with cetyltrimethylammonium bromide (CTAB) method, as reported in Mafra *et al* (2008), with some modifications. Next, 0.5 mL of CTAB extraction buffer (20 g CTAB/L, 1.4 M NaCl, 0.1 M Tris-HCl and 20 mM EDTA) was added and vortex or mixed thoroughly. Mixture was incubated for 1 hr at 65°C, with occasional stirring. The suspension was then centrifuged (10 min, 16,000g) and supernatant were collected. 200 µL of chloroform was added and vortex. Mixture was centrifuged (10 min, 14,000g) and the upper phase was transferred into a new tube containing double volume of CTAB precipitation solution (5 g/L, 0.04 M NaCl), the mixture was incubated for 1 hr at room temperature. After centrifugation (10 min, 14,000g), the supernatant was discarded and the precipitate was dissolved in 350 µL 1.2 M NaCl and mixed with 350 µL chloroform by vortex. The mixture was centrifuged (10 min, 14,000g) and the upper phase was transferred to a new tube containing same volume of isopropanol. Mixture was incubated overnight at -20°C. The next day, mixture was centrifuged (10 min, 14,000g) at 4°C, the supernatant was discarded and the pellet was washed with 500 µL of ethanol solution (70% v/v). After centrifugation (10 min, 15,000g), the supernatant was discarded carefully, the pellet was dried and the DNA was eluted in 100 µL of sterile ultrapure water. DNA purity and quantitation were measured by absorbance at Biophotometer (Eppendorf, Hamburg, Germany).

Table 1. Types of food and feed samples for the detection of *lectin* gene and target specific *cp4 epsps* gene

Products	Number of Samples	Positive to Lectin	Positive to EPSPS/RR
Raw soy bean	25	24	1
Processed food	41	34	10
Chocolate containing food	10	3	1
Vegetarian Food	2	2	2
Animal feeds			
Soybean hull pellet	1	1	1
Common animal feed	34	34	25
Rabbit pellet	4	4	0
Chicken feed	3	3	3
Dog feed	1	1	1
Pig pellet	1	1	1
Total	122	107	45

Oligonucleotide primers

Two different primer sets were used in this study. The primers LEC1 (GTG CTA CTG ACC AAG GCA AAC TCA GCA)/ LEC2 (GAG GGT TTT GGG GTG CCG TTT TCG TCA AC) were used to amplify endogenous *lectin* gene of the soybean the amplicon size of 164 bp (Angonesi Brod *et al*., 2007). Generally, *lectin* gene is useful to identify the amount of soybean genomic DNA in the samples (Yoshimura *et al*., 2005). However, the primers pairs EPSPS1 (GCC TCG TGT CGG AAA ACC CT)/ EPSPS3 (TTC GTA TCG GAG AGT TCG ATC TTC) targeting CP-4 enolpyruvylshikimate-3-phosphate synthase (*cp4-epsps*) gene that render herbicide tolerance gene in RRS, yielding an amplicon size of 118 bp (Matsuoka *et al*., 2002) was utilized for the detection and quantification of RRS.

Qualitative PCR condition

The duplex PCR system was performed using Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). Two sets of primers pairs LEC1/ LEC2 and EPSPS1/ EPSPS3 were used to detect the *lectin* gene and RRS in collected samples simultaneously. In duplex PCR reaction, a total reaction mixture volume of 20 µL containing of 2 µL of DNA extract, 1× PCR buffer (10 mM Tris-HCl (pH8.3), 50 nM KCl and 2 mM $MgCl_2$), 0.25 mM dNTPs, 1.5 units of i-*Taq*™ DNA polymerase (Intron, Gyeonggi-do, Korea), 0.125 µM of each primers of LEC1/ LEC2 and 0.25 µM of each primers EPSPS1/ EPSPS3. The PCR cycle program used in this study was: Predenaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min 30 sec, and a final extension of 5 min at 72°C.

After PCR amplification, 20 µL of amplified PCR product was resolved by 1.8% agarose gel electrophoresis and stained with ethidium bromide (0.5 µg/ml). The gel was visualized using a UV transilluminator (Alpha Imager, Alpha Innotech, USA).

Cloning

PCR reaction were carried out on genomic DNA extracted from 5% RRS from CRM with LEC1/ LEC2 primers and EPSPS1/ EPSPS3 primers respectively, yielded sufficient amount of the 164 bp fragment of soybean *lectin* gene and the 118 bp fragment of *cp4-epsps* gene, which were then subjected for cloning with Qiagen® PCR Cloning Kit (Qiagen, Hilden, Germany). The amplified DNA fragments were resolved by 1.8% agarose gel electrophoresis and the specific amplicons were purified from the gel by using Gene√All™ Gel Extraction SV kit (Seoul, Korea). Generated PCR amplicons was separately transformed into pDrive cloning vector (Qiagen, Hilden, Germany) according to manufacturer's protocol. Plasmid DNA were extracted from transformed bacterial cells using Fast Plasmid Mini Kit (Eppendorf AG, Hamburg, Germany) and finally, confirmed with PCR analysis and direct sequencing.

Construction of calibration curves

In order to quantify GMO contents of RRS with LightCycler® (Roche, Germany) system, two calibration curves were established with the endogenous *lectin* gene and the target specific *cp4-epsps* gene. The concentrations of plasmid DNA in ng/µL were measured by Biophotometer (Eppendorf, Hamburg, Germany). Tenfold dilution series were then made by diluting plasmid DNA with sterile ultrapure water, given concentration range of 0.013-13 ng/µl and 0.028 – 28 ng/µl for target specific *cp4-epsps* gene and endogenous *lectin* gene respectively. All of the PCR reactions were repeated three times. The calibration curves based on SYBR Green fluorescence were established by plotting Cp value against the logarithm of the plasmid DNA concentration in ng/µl. However, Cp value was defined as the cycler number that the amplification fluorescence signals above the threshold (Zhang *et al*., 2008). Threshold level is the amplification cycle at which a significant increase in fluorescence signal is first detected (Terry *et al*., 2001). 'Fit Point Method' was performed in quantification. Finally, the genomic DNA isolated from 5% RRS from CRM was utilized as external standard to validate the calibration curves.

Quantitative Real-time PCR

SYBR Green real-time PCR assays using Quantitect SYBR Green Kit (Qiagen, Hilden, Germany) were carried out in a LightCycler® 2.0 Instrument (Idaho Technology, USA, licensed to Roche Molecular Biochemicals, Mannheim, Germany), and the data was analyzed using the LightCycler® software version 4.05 (Roche, U.S.A.). A total volume of 20 µL consisted of 2 × QuantiTect SYBR Green mixtures, 0.5 µM of each primer, 1 µL of DNA and 7 µL of RNase-free water were filled in LightCycler® glass capillaries. The real-time PCR reactions were carried out with an initial denaturation for 15 min at 95°C, followed by 40 cycles of amplification and quantification with 15 s at 95°C, 10 s at 62°C and 10 s at 72°C. The specificity of primers can be determined according to the melting curve analysis. Thus, the PCR program was followed by melting curve program, during which the temperature was gradually raised from 72°C to 90°C at heating rate of 0.2°C/s with a continuous fluorescence measurement and lastly, cooling step to 40°C.

Determination of Roundup Ready Soybean content in unknown samples

In this study, the RRS content in unknown samples were determined with relative quantification methods, in which based on two different absolute quantifications of the endogenous gene and target specific gene. The genomic DNA of 5% RRS from CRM was used as a reference standard in absolute quantification methods of both assays. In this assay, endogenous gene, target specific gene and reference standard were filled in the separate glass capillaries and amplified independently during the same run.

According to the established calibration curves and the reference standard, the concentration (ng/µl) of the endogenous gene and target specific gene can be determined based on the cp value obtained from the analysis. As a result, the percentage of RRS content was calculated by dividing the concentration of the target specific gene by that of the endogenous gene and multiplying by 100%. Each of the samples was repeated twice. Standard deviations and relative standard deviation were calculated to estimate the precision of quantitative results (%).To ensure the reliability of this assay, the genomic DNA isolated from 5% RRS from CRM and sterile ultrapure water were used as positive control and negative control respectively.

Results and Discussion

Qualitative PCR analysis

In the case of large amount of samples, the CTAB DNA extraction is cost effective and able to extract amplifiable DNA from different type of samples. This study demonstrated 114 samples were successfully extracted and assayed by PCR. As shown in table 1, 107 out of 114 samples were detected for the presence of *lectin* gene (164 bp) and 45 samples were positive for the target specific *cp4-epsps* gene (118 bp). The PCR products of *lectin* and target specific *cp4-epsps* can be easily resolved in 1.8% agarose gel electrophoresis as shown in Figure 1.

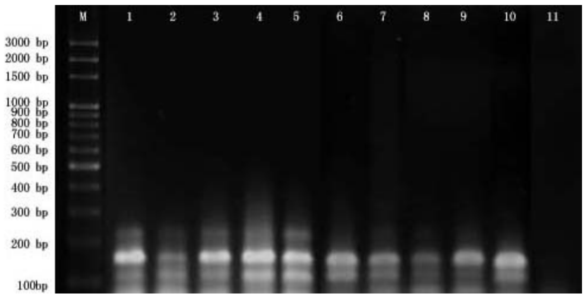

Figure 1. Qualitative PCR analysis of food and feed samples. Representative agarose gel electrophoresis of PCR product from food and feed samples with *lectin* gene (164 bp) and *cp4 epsps* gene (118 bp). Lanes: M, 100 bp ladder; 1, 5% RRS from CRM as positive control; 2, animal feed; 3 and 4, tofu samples; 5, processed food; 6, tofu samples; 7, processed food; 8, vegetarian food; 9 and 10, tofu samples; and 11, sterilized distilled water as negative control

The LEC1/LEC2 primers were used to confirm the occurrence of amplifiable soybean DNA in extracted samples. Our data exhibited that CTAB method is not suitable for DNA extraction for sample such as chocolate-containing food because only 30% of the samples DNA was successfully extracted. EPSPS1/ EPSPS3 primers set were used to identify the presence of the specific RRS content in the food samples.

In this study, 70% (31/44) animal feed samples were positive for RRS. Previously, Yoke-Kqueen (2006) from Malaysia also reported the occurrence of RRS in animal feeds that sold commercially, 92.3% (12/13) of the animal feed samples were contained RRS. Thus, results suggested that RRS can be detected in the animal feed. In the case of processed food samples that purchased commercially in Malaysian markets, 33.3% (13/39) of the samples were positive to RRS and among that, 8 samples were tofu. As reported in previous study, 21.2% (18/85) of the samples were positive for RRS and 44.4% were tofu (Abdullah, Radu, Hassan, and Hashim, 2006). Thus, consumers can easily obtained tofu containing GMO especially RRS in Malaysian markets.

Cloning and sequencing

In the PCR analysis with cloned plasmid DNA as template, the expected amplicons of *lectin* (164 bp) and *cp4-epsps* (118 bp) were visualized in agarose gel (data not shown). In addition, the sequences of inserted DNA (*lectin* gene and *cp4-epsps* gene) in plasmid DNA were validated by sequencing. BLAST results indicated that 90% and 93% homology with the *cp4 epsps* gene and 100% homology with the *lectin* gene, thus confirmed the inserted genes in the plasmid DNA.

Hence, both results from PCR analysis and sequencing suggested that cloned plasmid DNAs were suitable to be used as calibrators to set up the calibration curves for quantitative analysis of RRS content in the samples.

Setup of calibration curves

As described in previous studies, generally two choices of calibrators to constructs calibration curves, the genomic DNA from CRMs (Terry *et al*., 2001) and cloned plasmid DNA fragments (Zhang *et al*., 2008; Tavernier *et al*., 2001). However, Burns *et al*. (2006) revealed that plasmid DNA fragment was a good alternative to genomic DNA as a calibrant in GMO quantification. Besides that, there are some drawbacks of using genomic DNA extracted from CRMs, such as limited quantitative range is 0-5.0% GMO only, inconvenient preparation procedures and high cost (Zhang *et al*., 2008). As a result, cloned plasmid DNA fragments as calibrators will be a better choice as it provides an easy, cost efficient production, long-term stability, and more flexible alternative to genomic DNA extracted from CRMs (Tavernier *et al*., 2004).

For quantitative analysis, we have established two calibration curves, one with the cloned plasmid *lectin* gene fragment and the other with cloned plasmid *cp4-epsps* gene fragment. The PCR efficiency was determined from the slope of the

Table 2. Statistical accuracy for the quantitative system

5% RRS from CRM	True value (%)	Cp value *lectin*	Concentration	Cp value *epsps*	Concentration	Calculated RRS (%)	% of error/bias
Set 1	5	24.03	94.5	28.56	4.97	5.259	5.18
Set 2	5	24.43	98.8	29.76	4.53	4.585	8.3

Table 3. Quantitative analysis of the GM RRS content in food and feed samples

Positive sample	Matrix	Cp for lectin				Cp for epsps				% Roundup Ready[a]	Standard
		1	2	Mean Cp	SD	1	2	Mean Cp	SD	(mean value)	deviation (SD)
Raw Materials											
H2	Soild	23.23	23.09	23.16	0.099	37.21	36.90	37.06	0.219	0.05	0.004
Processed food											
9b	Solid	23.73	23.99	23.86	0.184	29.52	28.13	28.83	0.983	17.99	12.229
I8	Semi-solid	26.57	26.62	26.60	0.035	29.30	29.42	29.36	0.085	74.85	2.015
Tohu											
1g	Solid	25.02	24.93	24.98	0.064	31.14	31.26	31.20	0.085	8.56	0.806
7b	Solid	24.73	24.80	24.77	0.049	37.53	38.07	37.80	0.382	0.10	0.019
8b	Semi-solid	26.78	28.61	27.70	1.294	40.33	39.90	40.12	0.304	0.22	0.206
I7	Semi-solid	22.44	22.31	22.38	0.092	26.26	26.28	26.27	0.014	27.81	1.891
J2	Semi-solid	25.85	25.89	25.87	0.028	34.75	34.70	34.73	0.035	0.70	0.030
J3	Semi-solid	28.76	28.69	28.73	0.049	33.15	33.50	33.33	0.247	38.21	7.934
M2	Solid	30.31	29.80	30.06	0.361	37.03	37.06	37.05	0.021	9.11	2.373
M5	Semi-solid	29.50	30.58	30.04	0.764	38.70	39.22	38.96	0.368	3.07	1.307
Vegetarian Food											
L2	Semi-solid	27.65	27.79	27.72	0.099	29.63	29.73	29.68	0.071	59.44	1.524
L3	Semi-solid	26.06	26.06	26.06	0.000	28.03	28.77	28.40	0.523	32.78	0.844
Chocolate containing											
food 3b	Semi-solid	25.28	24.16	24.72	0.792		34.09	34.09		0.71	
Animal feeds											
Soybean hull Pellet											
Q	Solid	24.45	24.45	24.45	0.000	28.95	28.97	28.96	0.014	17.56	0.127
Common animal feed											
AF1	Solid	31.58	31.06	31.32	0.368	37.61	37.71	37.66	0.071	9.96	2.976
AF3	Solid	30.84	30.93	30.89	0.064	36.66	36.62	36.64	0.028	13.79	0.834
AF4	Solid	32.03	30.58	31.31	1.025	38.62	42.97	40.80	3.076	0.54	0.470
AF5	Solid	31.62	31.49	31.56	0.092	38.20	37.06	37.63	0.806	9.28	4.016
AF6	Solid	31.52	32.23	31.88	0.502	38.90	39.14	39.02	0.170	4.66	1.123
AF7	Solid	30.02	31.59	30.81	1.110	39.69	39.69	39.69	0.000	1.65	1.155
AF8	Solid	31.82	31.65	31.74	0.120	39.79	39.23	39.51	0.396	3.07	0.534
AF9	Solid	31.62	31.09	31.36	0.375	38.21	38.19	38.20	0.014	5.49	1.336
AF10	Solid	31.97	31.78	31.88	0.134	40.53	37.89	39.21	1.867	5.33	4.866
AF11	Solid	30.21	30.66	30.44	0.318	37.21	38.99	38.10	1.259	3.30	1.807
AF12	Solid	26.54	26.61	26.58	0.049	35.14	34.62	34.88	0.368	1.24	0.346
AF13	Solid	27.25	27.60	27.43	0.247	31.93	31.27	31.60	0.467	38.15	17.105
AF14	Solid	28.08	27.98	28.03	0.071	31.71	32.24	31.98	0.375	43.32	13.732
AF15	Solid	31.93	29.71	30.82	1.570	37.91	37.94	37.93	0.021	3.67	3.379
AF16	Solid	29.92	31.03	30.48	0.785	36.76	37.16	36.96	0.283	4.41	1.527
AF18	Solid	23.75	23.47	23.61	0.198	35.27	34.35	34.81	0.651	0.17	0.051
AF19	Solid	22.95	23.04	23.00	0.064	35.14	34.66	34.90	0.339	0.10	0.029
AF27	Solid	26.93	25.70	26.32	0.870	38.07	37.78	37.93	0.205	0.13	0.057
AF28	Solid	24.83	24.72	24.78	0.078	37.26	37.53	37.40	0.191	0.06	0.011
AF29	Solid	26.01	26.03	26.02	0.014	34.64	34.44	34.54	0.141	2.68	0.276
AF30	Solid	24.08	24.14	24.11	0.042	36.70	36.70	36.70	0.000	0.18	0.005
AF31	Solid	26.88	26.60	26.74	0.198	30.62	30.71	30.67	0.064	32.11	2.414
AF32	Solid	29.88	31.55	30.72	1.181	37.71	37.62	37.67	0.064	11.31	8.631
S	Solid	23.01	22.19	22.60	0.580	31.22	31.47	31.35	0.177	1.16	0.574
K3	Solid	30.95	31.22	31.09	0.191	37.47	36.93	37.20	0.382	11.43	4.172
Chicken Feed											
A	Solid	23.72	23.94	23.83	0.156	27.22	27.51	27.37	0.205	31.02	0.495
E	Solid	24.00	23.89	23.95	0.078	27.64	27.77	27.71	0.092	27.12	3.048
F	Solid	26.23	26.45	26.34	0.156	29.89	29.85	29.87	0.028	50.77	6.512
Pig Feed											
R	Solid	23.12	23.30	23.21	0.127	27.88	27.86	27.87	0.014	14.73	1.421
Dog Feed											
AF33	Solid	25.27	24.66	24.97	0.431	29.51				25.69	

[a]The percentage of RRS is calculated as: $\dfrac{\text{target specific gene concentration}}{\text{soybean lectin gene concentration}} \times 100\%$

curve. The generated calibration curves generate PCR efficiencies of endogenous gene and target specific gene with the value 1.948 and 1.738 respectively, closed to 2. Additionally, the errors of the calibration curves were 0.0171 and 0.029 for endogenous gene and target specific gene respectively. The high PCR efficiency and low error value indicated the generated calibration curves are suitable for GMO quantitative analysis (Zhang *et al*., 2008). Besides, the 5% RRS from CRM were also used to evaluate the accuracy of established quantitative system. The accuracy of the quantitative results was calculated by the error between the calculated % of RRS content and true value of 5%. As described by various researchers previously, the errors of the quantitative results were ranged 0.67 to 28.00% (Yang *et al*., 2007) and 0.60 to 8.78% (Zhang *et al*., 2008). In this study, the measured percentage of RRS was 5.23% and 4.59% with the errors of 5.18% and 8.30% in which below 20.00% as shown in table 2. Thus, this system is suitable for GMO quantitative analysis.

Quantitative Real-time PCR analysis

According to Hahnen *et al*. (2002), real-time PCR is widely recognized as the most sensitive that gives a reproducible results and large dynamic range. GMO quantitative analysis using real-time PCR becomes crucial especially to fulfill the labeling legislation of GMO product. Analysis using LightCycler® system will allow PCR amplification complete in less than 2 hour (Tavernier *et al*., 2001). When comparing to the most common real-time assays such as TaqMan, minor groove binding, molecular beacons and SYBR Green assays, SYBR Green is the most economical assay compared to other assays (Andersen *et al*., 2006).

The measurement of GMO material was calculated as percentage genome/genome or percentage weight/weight (Bonfini *et al*, 2001). In this study, the quantitative analysis of the RRS content in the food samples were found similar to the study by Taverniers *et al*., 2001. The relative quantification methods were applied based on the two different absolute quantifications of the endogenous and target specific gene.

In the present study, 45 samples showed positive for qualitative PCR and subjected to quantitative real-time PCR. The detection of *lectin* gene in this study gave an average Cp values varied from 22.38-31.88 cycles with the standard deviation of 0.00-1.57 while Cp for the RRS that correlated with the amount of GM content present in each standard ranging from 26.27 – 40.80 cycles with a standard deviation of 0.00-3.076 as shown in table 2. The standard deviations of this data obtained support the reproducibility and stability of LightCycler® Real-time PCR system.

According to the quantitative results reported in table 3, 11 samples were found contained <0.9% with RRS, 4 samples ranged 0.9% to 3%, 6 samples ranged 3% to 5%, another 6 samples ranged between 5-10% and 18 samples were showed >10%. The data obtained showed that 40% of the samples that collected from the Malaysian market contain very high percentage of RRS content. Based on the 0.9% threshold level established by EU labeling legislation, 76.5% (34/45) samples in this study were detected more than 0.9% of the RRS content and should be labeled 'GMO' product. By using the SYBR Green assay in our quantitative system, the primers that we used were highly specific corresponding to the melting curve showed only one peak per primer set (Figure 2). Various researchers worldwide reported that SYBR Green assays demonstrated extremely high sensitive for the short amplicon and the amplification products exhibit cleanest melting curve (Andersen *et al*., 2006).

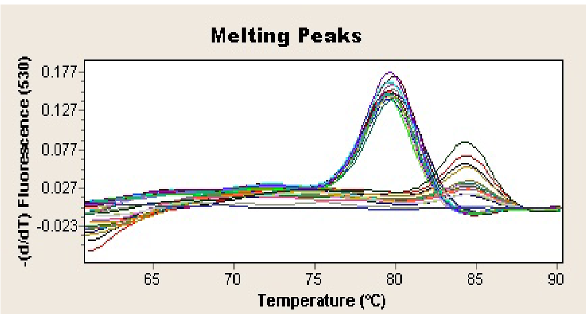

Figure 2. Melting Peaks curves generated from the SYBR Green assay in LightCycler® real-time PCR system using LEC1/LEC2 primers and EPSPS1/EPSPS3 primers

Besides that, there are several studies reported the presence of RRS content in the food samples commercially available in the market at few countries. There were 64.5% (40/62) soy derived product were tested positive for RRS and two samples (1.2% and 1.7%) revealed GMO content above the threshold set by Brazilian Legislation (Angonesi Brod and Arisi, 2008). In Brazil, 17% (34/200) of the soy derived products were detected contain RRS, five samples contain <1% RR soy, eight samples 1-4%, ten samples 4-10% and eleven sample >10% (Greiner *et al*., 2005). Zhou *et al*. (2007), found that one sample out of 60 samples containing RRS in open market from Shenzhen city. Besides, 20% (8/40) food samples collected from Egyptian markets were found containing RRS (El Sanhoty *et al*., 2002).

This study gave an insight of the occurrence of different percentages of RRS content in the food samples and animal feeds that are sold commercially in the Malaysian market. As a result, labeling legislation in Malaysia becomes crucial not only had to increase consumers' awareness of food contained GMO derivatives, but also giving them an opportunity to make an informed choice.

Conclusion

In conclusion, the CTAB DNA extraction methods, qualitative PCR and quantitative real-time PCR are suitable to detect and quantitate GMO content in food and feed samples and practically useful especially in the case of huge amount of samples due to its cost effectiveness. However, the established quantitative analysis of GMO content by using SYBR Green LightCycler® Real-time PCR coupled with melting curve analysis provide a fast, reproducible and sensitive quantitative approach. Since Malaysia will be implementing mandatory labeling food ingredients containing GMO derivatives soon, genthe quantitative approached as described in this study will facilitate the labeling process.

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