Wageningen Academic Publishers

Single laboratory method performance evaluation for the analysis of Roundup Ready® soy flour by qualitative and quantitative detection methods

R. Yılmaz[1*], C. Bayraç[2] and M. Yücel[3]

[1]Department of Food Engineering, Beytepe Campus, Hacettepe University, 06800 Ankara, Turkey; [2]Department of Bioengineering, Karamanoğlu Mehmetbey University, Yunus Emre Campus, 70100 Karaman, Turkey; [3]Central Laboratory, Molecular Biology and Biotechnology R&D Center, Middle East Technical University, 06800 Ankara, Turkey; remziye@hacettepe.edu.tr

Received: 25 July 2016 / Accepted: 6 January 2017

RESEARCH ARTICLE

Abstract

The Republic of Turkey has approved 7 soybean and 25 corn genetically modified (GM) events for animal feed use only and the biosafety legislation has banned the cultivation of GM crops and requires that all genetically modified organisms (GMOs), including imports, to be approved for use and further establishes a strict policy of testing for food, feed and seed potentially containing GMOs. For the GMO analysis, each laboratory should establish the verification on method performance criteria and calculation of measurement uncertainty. The aim of this study is to define the verification of qualitative and quantitative detection of Roundup Ready® soybean as a model for single laboratory verification in the context of the European Network of GMOs Laboratories guidance documents. First, two methods were used for the extraction of nucleic acids (DNA) and their extraction efficiencies were compared based on the quantity, purity, fragmentation state of DNA and inhibition in polymerase chain reaction (PCR). Second, a verification procedure of a real time PCR method for qualitative detection of cauliflower mosaic virus *35S* promoter and *Agrobacterium tumefaciens Tnos* sequences in DNA samples extracted from certified reference materials and GM soy flour samples was performed. Last, the standard curves were prepared in order to explain verification of quantitative real-time PCR analysis by reaching the ideal value of -3.62 for *lec* reference gene and -3.40 for *A. tumefaciens* strain CP4 5-enolpyruvulshikimate-3-phosphate synthase (EPSPS*)* enzyme target gene. All method performance criteria for quantification (within-laboratory reproducibility standard deviation, relative standard deviation, uncertainty, bias, limit of detection, limit of quantification, and linearity) were met and thus the method in this study was verified. Finally, the document highlights a clear example for analysis of GMOs in food and feed samples, and points out the need for interlaboratory studies at the national and international level.

Keywords: DNA, *35S* promoter, Tnos, CP4 EPSPS, real-time PCR, verification

1. Introduction

Genetically modified (GM) crops are alternatives that can contribute to solving issues related to climate change, global warming, and a lack of food and animal feed (Lim *et al.*, 2016). The soybean (*Glycine max*) is the most important genetically modified crop from which 81% of the world's planted area corresponds to the Roundup Ready® soybeans (RRS) (Kamle and Ali, 2013). Its development was based on recombinant DNA technology through the introduction of a glyphosate tolerant form of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene isolated from *Agrobacterium tumefaciens* strain CP4 (Querci *et al.*, 2006). Roundup Ready® soybean event with trade name GTS 40-3-2 (unique identifier MON-04032-6) is one of the approved GM soy events in the European Union (EU) and Turkey with strict mandatory labelling regulations at 0.9% threshold level for approved products and 0.5% for products that have not yet been approved for the presence of GM crop ingredients (Brooks, 2012; GAIN, 2016; Masip *et al.*, 2013).

Verifying qualitative and quantitative detection methods and estimation of measurement uncertainty are used by the laboratories to enhance their system in quality and technical operations. The consensus documents for the validation and verification of genetically modified organism (GMO) analysis including qualitative and quantitative assays are being published by the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF; http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). The Network of GMO Laboratories (ENGL) has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the 40-3-2 transformation event in soybean DNA (Mazzara *et al.*, 2007). However, little documentation of method performance parameters is available using this method as a clear example for a single laboratory. In order to verify the assay, we describe here, in-house verification of DNA extraction, qualitative and quantitative detection methods for GMO testing of RRS flour according to the EU research centres on GMOs guidance documents is warranted (Mazzara *et al.*, 2007; Trapman *et al.*, 2009).

The market still faces uncertainty over the monitoring and labelling of GM products in European countries and Turkey. For the detection and quantification of transgenic crops, reference methods are provided by applicants in the EU for authorisation and they are validated by EU-RL GMFF, assisted by ENGL. Reliable and efficient methods for detecting GMOs will be essential for establishing an effective system for traceability all throughout the supply chain from seed producers to final consumers. Performance of the methods used in laboratories around the world should be uniform in order to obtain reliable and comparable results. There are some indecipherable problems on the reproducibility obtained from distinct experimental trials from the official laboratories for GM analysis in Turkey. However, little documentation on method performance parameters is available in using the method for other food matrices.

2. Materials and methods

Materials

Certified soy reference materials (CRM); GTS 40-3-2 Blank (ERM-BF410ak), 10% (ERM-BF410dk) and 100% (w/w) (ERM-BF410gk) were supplied from EC JRC Institute for Reference Materials and Measurements (IRRM, Luxembourg, Luxembourg). Soy flour samples with unknown GM content from routine analysis were also used for DNA isolation and real time polymerase chain reaction (RT-PCR) quantification. All chemicals used for the preparation of extraction solutions were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The foodproof® GMO sample preparation kit, foodproof® GMO screening kit and foodproof® GMO Soya Quantification Kit were supplied by Biotecon Diagnostics (Potsdam, Germany).

Methods

Preparation of samples

As starting material of soy flour samples, 200 grams of unknown sample materials were separated into two equal homogeneous parts. One part was stored at -20 °C as a stock sample. The representatives of the other part were weighed as 100 mg in aseptic conditions in order to prevent cross contamination between CRM and soy flour samples.

Genomic DNA extraction

Genomic DNA was extracted from CRM and homogenised soy flour samples according to the hexadecyltrimethyl-ammonium bromide (CTAB)-based method reported in ISO 21571:2005 (ISO, 2005b) and EC (2014). As an alternative method, a commercial kit (foodproof® GMO sample preparation kit; Biotecon Diagnostics) was used for the extraction of genomic DNA. All genomic DNA was analysed by agarose gel electrophoresis by using 1.0% agarose gels containing 0.5 µg/ml of GelRed dye in 1 × Tris-Acetate-EDTA buffer at 100 V (Biorad Sub-Cell® GT, Bio-Rad Laboratories, Hercules, CA, USA). DNA concentrations were determined spectrophotometrically.

PCR inhibition control

DNA extracted from CRM (GTS-40-3-2; 100%) were serially diluted fourfold (1:4, 1:16, 1:64, 1:256, and 1:1,024) and the taxon specific *lectin* gene was amplified with RT-PCR (LightCycler 1.5 Real Time PCR; Roche Molecular Systems, Inc., Basel, Switzerland) (10 min at 95 °C for denaturation, 45 cycles of 95 °C for 10 s, 60 °C for 20 s, 72 °C for 10 s and cooling at 40 °C for 30 s). The primer/hybridisation probe set within the content of the commercial kit was used for the amplification of native lectin gene in order to show the integrity of DNA. For each dilution, amplification was performed in triplicates and the mean cycle threshold (Ct) values were plotted against a log of dilutions.

Qualitative analysis

In qualitative analysis, DNA amplification was carried out by RT-PCR with 50 ng/µl of DNA. The cycling condition was as follows: 15 min at 95 °C for denaturation, 45 cycles of 95 °C for 10 s, 60 °C for 25 s, 72 °C for 10 s and cooling at 40 °C for 30 s. For each sample, three biological replicates were used in two technical replicates.

Quantitative analysis

For identification and quantification of RRS event in soy samples, foodproof® GMO Soya Quantification Kit (Biotecon Diagnostics) was used with same carousel-based system. Within the content of the kit, the reference gene was referred to as a fragment of the lectin gene of soya and its amplification was performed with sequence specific primers and hybridisation prob set. For the soya GMO gene, a fragment of the *35S*-promoter sequence of the cauliflower mosaic virus and the downstream located chloroplast transit signal sequence of *Petunia hybrida* were selected as targets for the amplification, thus, specific primers and a hybridisation prob set were used in reaction mix. The standard curves for both the reference gene and GM target gene were constructed with calibrator DNA and its diluted samples (dilutions of 1:4, 1:16, 1:64, 1:256, and 1:1,024). DNA amplification was carried out in a final volume of 20 µl containing enzyme mix, soy reference gene mix or soy GM gene detection mix and DNA (50 ng/µl) solution. The cycling condition was as follows: 10 min at 95 °C for initial denaturation, 45 cycles of amplification at 95 °C for 10 s, 60 °C for 20 s, 72 °C for 10 s and cooling at 40 °C for 30 s.

Two replicates for each dilution were analysed and standard curves were constructed for the reference gene and RRS gene. The quantitative analyses of the target gene were determined by using the relative quantification method and the percentage of RRS relative to the total soya content within the sample of interest. The relative amount of the target gene and reference gene was determined for each sample and one calibrator, integrated in each LightCycler run. For the calculation of the final result only the crossing point (CP) values obtained by the LightCycler analysis software were used that the relative ratio of a target gene is computed. It is based on real-time PCR efficiencies (E) and the crossing point difference (Δ) of an unknown sample versus a control ($\Delta CP_{control - sample}$) (Pfaffl *et al.*, 2002).

Data analysis

DNA concentration, yield and repeatability was calculated for each of the DNA isolation methods. Yield and average DNA extraction efficiency were represented by average concentration of DNA and the ratio of average DNA concentration to total isolation number, respectively.

The efficiency of two extraction methods was compared based on T statistical test for independent samples at 95% confidence interval. Also, relative repeatability standard deviation (RSD_R) for both methods was obtained. For the inhibition control, the extrapolated Ct values for each dilution were calculated by linear regression from the curve of the mean measured Ct values against log of dilutions. False positive and negative rates were calculated for qualitative analysis method along with the positive and negative predictive values.

RT-PCR results were analysed by relative quantification method and the measurement uncertainty was obtained. The method acceptance criteria; trueness, amplification efficiency, R^2, RSD_R, limit of quantification (LOQ), limit of detection (LOD) and linearity were determined as the results (Trapman *et al.*, 2009).

3. Results and discussion

Efficiency of DNA extraction methods

High quality DNA is required for accurate qualitative and quantitative GMO analysis with RT-PCR. In this study, two commonly used DNA extraction techniques were compared and their suitability for qualitative and quantitative analysis was assessed. The effect of sample matrix on nucleic acid quantification was assessed by comparing CRMs at 3 different GM content and 3 soybean matrixes with unknown GM content. For each sample material, extraction process was performed in triplicates. The amount of DNA needed for reliable amplification and extraction efficiency could be identified as the crucial parameters. Therefore, it was chosen as the primary criterion by which to evaluate the quality and performance on different matrixes and extraction techniques. The concentration of DNA extracts and their purity were determined spectrophotometrically according to a method described in ISO 21571:2005 (ISO, 2005b). In order to show the repeatability of measurement of DNA concentration via spectrophotometer, the absorbance and concentration values of standard DNA sample (100 ng/µl) was measured in triplicates. The mean concentration of the standard DNA sample was 99.87 ng/µl with a standard deviation of 0.24 (n=3). When compared with expected value of standard DNA, the percentage difference between the actual and measured concentrations was calculated as 0.13%. Therefore, it was demonstrated that the quantification method via spectrophotometer for DNA extracted with two different methods was reliable and repeatable.

For DNA samples with absorbance values higher than 1 at 260 nm, they were diluted to be quantified accurately. Since maximum absorbance of CTAB solution is 260 nm, spectrophotometric measurement of DNA extracted with CTAB-based method was evaluated with absorbance value of this solution to prevent its interference with the detection of pure DNA. As shown in Table 1, total genomic DNA concentrations of most samples extracted with two different techniques were within the range of approximately 0.2 to 0.6 µg/µl. With the CTAB-based method, DNA extracted from soy flour samples showed variety among three samples. This could be explained with the differences in particle size of sample materials. Nevertheless, the DNA

Table 1. Average DNA concentrations and extraction efficiencies of certified soy reference materials at three different genetically modified concentrations (GTS 40-3-2 Soy Blank, 10% and 100%) and soy flour samples.

Sample material	Method[1]	Average DNA concentration (ng/µl)	Average DNA extraction efficiency (ng/µl)	RSD_R (%)[2]	Coefficient of variation (%)
Soy Blank	CTAB-based method	257.10	128.55	0.59	0.72
Soy 10%		280.60	140.30	0.44	0.54
Soy 100%		310.20	155.10	0.13	9.71
Soy Blank	Kit	350.60	87.65	0.24	0.30
Soy 10%		255.60	63.90	0.52	0.64
Soy 100%		345.00	86.25	1.83	2.25
Sample A	CTAB-based method	232.69	116.35	6.18	11.97
Sample B		186.83	93.42	10.32	21.41
Sample C		358.46	179.23	9.29	20.01
Sample A	Kit	575.33	143.83	3.40	6.55
Sample B		526.33	131.58	2.83	4.67
Sample C		552.22	138.06	4.42	7.91

[1] Two different DNA extraction methods were used: the hexadecyltrimethylammonium bromide (CTAB)-based method (ISO, 2005b and EC, 2014) and methods given by the manufacturer of the foodproof® GMO sample preparation kit (Biotecon Diagnostics, Potsdam, Germany).
[2] RSD_R = relative repeatability standard deviation.

concentration measured was higher than the working concentration described in the qualitative and quantitative protocols of RT-PCR. For the comparison of two extraction techniques, T statistical test was applied on the average DNA extraction efficiency and results showed that there was not any significant (P>0.05) difference between the amounts of DNA extracted by these methods. Also, the purities of DNA extracted by these two methods were also compared based on the ratio of OD at 260 nm to 280 nm. All DNA samples showed purity within the range of 1.8-2.1 meaning that they were free of protein contamination. When these results were compared with the 'Report on the validation of a DNA Extraction method for soybean seeds' (EC, 2008) published by the European Commission Joint Research Center (EC, 2014), it could be stated that the results of DNA extraction were applicable to qualitative and quantitative testing by RT-PCR in further studies. According to the report, the mean concentration of DNA sample extracted from soybean seeds via CTAB method was 439.8 ng/µl with standard deviation and coefficient of variation of 43.2 ng/µl and 9.8%, respectively. However, in this study, standard deviation and coefficient of variation were calculated as 21.16 ng/µl and 0.54%, respectively, for 280.60 ng/µl of the mean concentration of DNA.

Documentation of fragmentation

Fragmentation of genomic DNA is another important handicap for obtaining high quality DNA. The steps in a manual extraction procedure or kit procedure can damage DNA physically with shearing so that amplification of fragmented DNA can be inhibited completely or the amplification efficiency can decrease. Agarose gel electrophoresis provides information about the fragmentation as a routine method. Beside fragmentation, this technique also allows us to evaluate the presence of RNA or other contaminants in extracted DNA samples. In the present study, gel results showed that there was no fragmentation in DNA extracted by both methods (Figure 1). The molecular weights of extracted DNA samples were higher than the expected amplicon size as in agreement with RT-PCR studies. Moreover, extracted DNA samples were not contaminated by RNA molecules. Only one intact DNA band was observed for each of the extracted DNA samples on gel results.

PCR inhibition control

For the inhibition control, the amplification reaction was set for samples serially diluted from a standard soybean DNA. Based on the shift in measured quantification cycle (Ct) relative to extrapolated Ct, inhibition in the amplification reaction was evaluated for extracted DNA samples (Waiblinger and Grohmann, 2014). Generally, for routine RT-PCR analysis, the amplification of internal positive control is a sign for the presence or loss/degradation of the target sequence during processing. However, for GMO analysis, taxon specific or plant specific sequences are amplified for the inhibition test. In this study, DNA extracted from CRM (GTS 40-3-2, 100%) was diluted fourfold serially and amplified for the detection of the taxon specific gene of soy and lectin *(lec)* gene. Mean measured

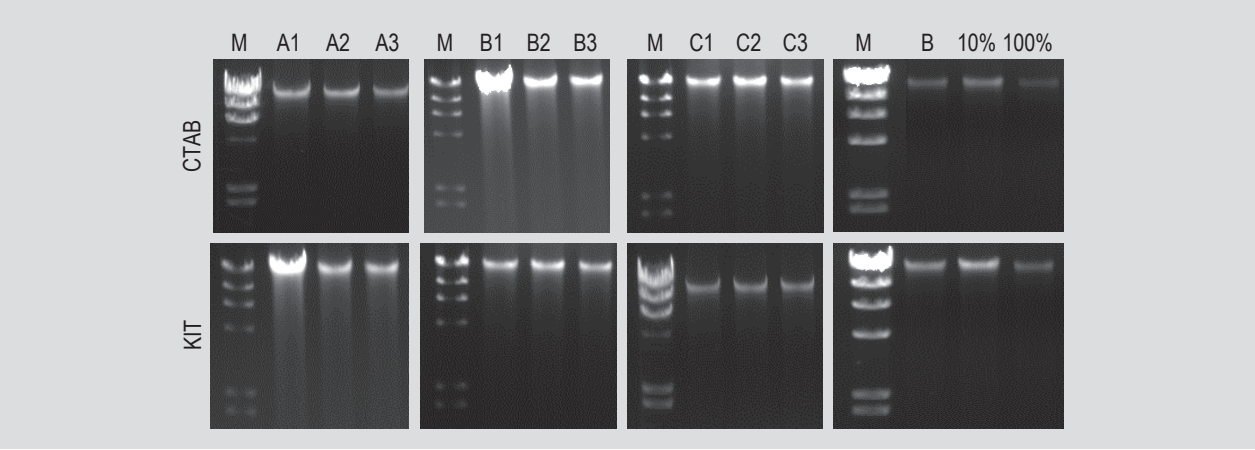

Figure 1. Agarose gel electrophoresis results of nine genomic DNA samples extracted with the hexadecyltrimethylammonium bromide (CTAB)-based method or methods given by the manufacturer of the foodproof® GMO sample preparation kit (Biotecon Diagnostics, Potsdam, Germany) from soybean samples (A, B, C) and six genomic DNA samples extracted from certified soy reference materials (B = blank, 10% and 100%; M = Lambda DNA/HindIII Marker).

Ct values for DNA samples were plotted against the log of dilutions and the slope of the curve was found as -3.496 (R^2=0.999). Based on the slope of curve, Ct values were extrapolated for each dilution and the absolute differences between the measured and the extrapolated Ct values (ΔCt) were compared in order to control the inhibition in PCR. As shown in Figure 2, for each dilution, extrapolated and measured Ct values were not significantly different from each other (P>0.05). According to the standards stating that ΔCt of the dilutions should be below 0.5 and the slope should be in the range of -3.6 to -3.1, PCR inhibition in this study was not detectable. ΔCt values for each dilution were smaller than 0.5 with the slope of -3.496.

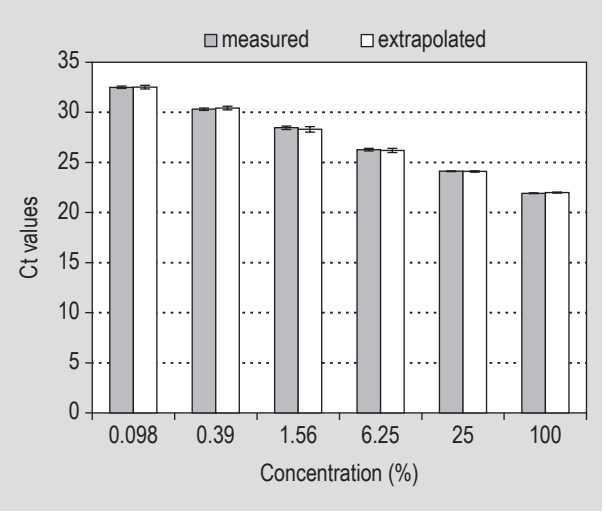

Figure 2. The comparison of measured quantification cycle (Ct) values and extrapolated Ct values of soya *lectin* gene for genetically modified (GM) soy with different GM contents (percentage concentration values for all dilutions).

Qualitative analysis

In this study, a verification procedure of a RT-PCR method for qualitative detection of *p35S* and *tnos* sequence in DNA samples was extracted from CRM and soy flour. According to the gene cassette given by Monsanto (2000), plasmid constructed for transformation contains *P-35S* as promoter, *CP4EPSPS* gene for glyphosate tolerance, *gus* gene as selective marker, *nptII* gene for antibiotic resistance and *T-nos* as terminator. Based on this, the presence of RRS 40-3-2 was confirmed qualitatively by the amplification of promoter and terminator region in the present study. The Ctvalues for the *p35S* and *tnos* for all DNA samples were the mean of three replicates (data not shown here).

The false-negative rate is the probability that a known positive test sample has been classified as negative by the method. However, the false-positive rate is the probability that a known negative test sample has been classified as positive by the method. Being method performance criteria they were calculated as follows (ISO 21098; ISO, 2005a):

$$R_{fn} = \frac{M_p}{N_p} \times 100\%$$

$$R_{fp} = \frac{M_n}{N_n} \times 100\%$$

Where R_{fn} is the false negative rate, M_p is the number of misclassified known positives, N_p is the total number of positive test samples, R_{fp} is the false-positive rate, M_n is the number of misclassified known negatives and N_n is the total number of negative test samples.

It was observed from the amplification curves (data not shown here), both reference gene and p*35S/tnos* genes were positive for all replicates. Due to the absence of misclassified

known negatives, the false-negative rate for sample A was calculated as zero. Since *p35S* and *tnos* were detected quantitatively, this sample could have a potential to be RR soy, thus, a further characterisation study was performed in quantitative analysis.

However, both *p35S* and t*nos* genes were negative in all replicates of sample B. The false-positive rate was calculated as zero since there were no misclassified known positives. Since both genes were absent, further characterisation and quantification studies for this sample were not performed in this study.

Importantly, the qualitative method worked in an accuracy manner with an acceptable precision in a limited range of concentrations and thus the false positive and negative results ensured the method performance criteria in this study.

Quantification

Standard curves

The standard curves of both *CP4EPSPS* gene and *lec* gene were constructed with calibrator DNA at serial dilutions. According to the criteria reported by ENGL (2008), the amplification efficiency should be -3.32 theoretically with an efficiency of 100% in each cycle of amplification in reaction. Thus, the average slope of the regression line should be within -3.1 and -3.6 and for this study it reached the ideal value of -3.62 for the reference gene (y = -3.62x + 29.15) and -3.40 for the target gene (y = -3.40x + 28.82). The linearity of reactions was very high, as R^2 coefficient was 0.99 for both target gene and reference gene assays.

Reproducibility analyses within-laboratory

The first step of verification consisted of verifying that all RT-PCR assays showed an acceptable efficiency and linearity, in agreement with the requirements of the ENGL (2008). In order to evaluate within-laboratory reproducibility, three CRMs of soy containing low, medium and high concentration of RR event were analysed independently. For each concentration of materials, two biological replicates were used in two parallels and GM event concentrations were evaluated to calculate the within-laboratory reproducibility standard deviation (S_{RL}) and relative standard deviation (RSD_R). Measurement results were given in Table 2.

For the calculation of S_{RL}, mean value of difference between two parallel measured results belonging to material having lowest GM concentration (d) and constant depending on the number of independent measurements (n) were used as follows

$$S_{RL} = \frac{d}{n} = \frac{0.034}{2.059} = 0.017 \text{ (in the case of four independent measurement)}$$

Measurements, beside once used for the calculation of S_{RL} were evaluated for RSD_R. RSD_R is calculated from the division of average relative differences (rad) to constant depending on n. The accepted criterion for RSD_R is below 25% according to the criteria reported by ENGL (2008). The performance of the method used in this study provided an acceptable value of relative standard deviation for the tested DNA samples, thus, the repeatability conditions were precise.

$$RSD_R = \frac{rad}{n} = \frac{9.774}{2.059} = 4.747$$

Bias control

CRMs containing medium and high concentration of GM event were analysed for the bias control. Four measurements were carried out independently and the results were represented in Table 3.

Table 2. Measurement results of certified soy reference materials at three genetically modified (GM) concentrations (GTS 40-3-2 Soy Blank, 10% and 100%).

Sample	Measured GM concentration, c_1	Measured GM concentration, c_2	Mean, ci	Difference[1], di	Relative difference, radi
Soy Blank	0.089	0.153	0.121	0.065	53.269
Soy Blank	0.037	0.040	0.038	0.003	8.830
Soy 10%	10.128	9.881	10.005	0.247	2.468
Soy 10%	12.917	12.672	12.795	0.245	1.913
Soy 100%	96.500	87.921	92.210	8.579	9.304
Soy 100%	127.647	98.868	113.258	28.779	25.410

[1] Mean difference, d, and rad (%) calculated as 0.034 and 9.774, respectively.

Table 3. Measurement results of certified reference materials at three different genetically modified (GM) concentrations (GTS 40-3-2 Soy 10% and 100%).

Sample	GM concentration, c (g/kg)	Mean GM concentration, c_m (g/kg)	Standard deviation, s (g/kg)
Soya 10%	10.128	11.400	1.617
Soya 10%	9.881		
Soya 10%	12.917		
Soya 10%	12.672		
Soya 100%	96.500	102.734	17.262
Soya 100%	87.921		
Soya 100%	127.647		
Soya 100%	98.868		

CRMs used in this study had certain certified GM concentration and expanded uncertainty (U_{CRM}) value stated as follows:

GTS 40-3-2 Soy 10: 10±1.0 g/kg

GTS 40-3-2 Soy 100: 100±7.0 g/kg

As mentioned in the certificate of reference materials, a coverage factor of k=2 was applied with the confidence interval of 95% and therefore, uncertainty (u_{CRM}) of each reference material was equal to division of expanded uncertainty to coverage factor. u_{CRM} of GTS 40-3-2 Soya 10% and GTS 40-3-2 Soya 100% were 0.5 g/kg and 3.5 g/kg, respectively. In order to compare the average of measurements with the value stated at certificate for each concentration, standard deviations (s) were divided by the square root of n. As a result, the uncertainty of measurement (u_m) was calculated for each concentration of certified material. As in this study; $u_{m(GTS\ 40\text{-}3\text{-}2\ Soya\ 10)}$ and $u_{m(GTS\ 40\text{-}3\text{-}2\ Soya\ 100)}$ were 0.809 g/kg and 8.631 g/kg, respectively.

After the measurements of CRMs at three GM concentrations and the calculations of u_m, absolute differences (Δ_m) between mean measured value (c_m) and certified value (c_{CRM}) were estimated by the following equations:

$$\Delta_{m(GTS\ 40\text{-}3\text{-}2\ Soya\ 10)} = |c_m - c_{CRM}| = 1.4\ g/kg$$

$$\Delta_{m(GTS\ 40\text{-}3\text{-}2\ Soya\ 100)} = |c_m - c_{CRM}| = 2.734\ g/kg$$

The uncertainty values of absolute difference (u_Δ) were evaluated from uncertainty of certified value (u_{CRM}) and measurement uncertainty (u_m):

$$u_\Delta = \sqrt{um^2 + ucrm^2}$$

u_Δ was calculated as 0.951 for GTS 40-3-2 Soya 10 and 9.314 for GTS 40-3-2 Soya 100. u_Δ was multiplied by coverage factor k=2 with the confidence interval of 95% in order to calculate the expanded uncertainty values (U_Δ). For GTS 40-3-2 Soya 10, U_Δ was 1.902 and for GTS 40-3-2 Soya 100, U_Δ was 18.628.

Calculated U_Δ and Δ_m between mean measured value and certified value were compared for the control of bias and if Δ_m was found to be equal to or smaller than U_Δ then there was no bias in analyses method for that study. It means that there is no significant difference between measured value and certified value for material within that method. For this study:

- $\Delta_m \leq U_\Delta$; method did not have bias at any concentration level of certified materials.
- For GTS 40-3-2 Soya 10 $\Delta_m = 1.4 \leq U_\Delta = 1.902$.
- For GTS 40-3-2 Soya 100 $\Delta_m = 2.734 \leq U_\Delta = 18.628$.

In addition, this study presented the correlation of uncertainty values between CRMs at different GM content. As observed from U_Δ of CRM GTS 40-3-2 at the GM content of 10% (1.902) and that at the GM content of 100% (18.628), with the increase in GM content, uncertainty values increased at the same rate. Therefore, for same CRM at different GM contents, U_Δ could be estimated from this correlation. For example, U_Δ of CRM GTS 40-3-2 at the GM content of 1% could be assumed as in the range of 0.0186 to 0.19.

For the estimation of uncertainty components associated to the bias; the following equations were used:

$$\text{Relative bias } (bias_r) = \frac{c}{CCRM} = 1.14$$

$$U_{biasr} = \sqrt{\frac{RSDR^2}{n} + \left(\frac{UCRM \times 100}{CCRM}\right)^2} = 4.229$$

Relative standard uncertainty (RSU) =

$$\sqrt{RSDR^2 + Ubiasr^2} = \% 6.357$$

Absolute bias ($bias_a$) = $c - c_{CRM} = 1.4$

$$u_{biasa} = \sqrt{\frac{SRL^2 + (c + RSDRL)^2}{n} + uCRM^2} = 8.089$$

Absolute standard uncertainty (u_o) =

$$\sqrt{SRL^2 + Ubiasa^2} = 8.089$$

Determination of detection and quantification limits

LOD is explained as 'minimum amount or concentration of the analyte in a test sample which can be detected reliably but not necessarily quantified' whereas LOQ is 'the lowest concentration or amount of the analyte in a test sample which can be quantitatively determined with an acceptable level of precision and accuracy' in ISO 24276:2006 (ISO, 2006).

LOD is generally expressed as the amount of analyte at which the analytical method detects the presence of the analyte at least 95% of the time. It is given by following formula and it was calculated as 0.201 for this study:

$$LOD = \frac{4Uo}{1 - (4RSU^2)}$$

Calculated LOD value represented absolute LOD (LOD_{abs}) of the method for the detection of RRS with PCR method explained above. Practical LOD ($LOD_{pract)}$ could be also calculated from the ratio of LOD_{abs} to measured reference gene amount and expressed in percentage unit (%).

For the calculation of LOQ, beside u_o and RSU, the largest acceptable relative standard uncertainty (RSU_{max}) was also used and calculated as 0.235 in this study:

$$LOQ = \sqrt{\frac{Uo^2}{RSUmax^2 - RSU^2}}$$

Linearity

For linearity analysis, DNA extracted from CRM with 100% GM content was diluted at six serial concentrations and two replicates for each dilution were analysed for their amplification in RT-PCR. As shown in Figure 3, the mean measured concentration values were correlated with the known concentration values of each dilution. The slope of the linearity graph of measured versus known concentrations was 1.004 (R^2 of 0.999). The measured values of six dilutions were strongly correlated with known values. This result indicated that the accuracy of the measurement was ideal and credible.

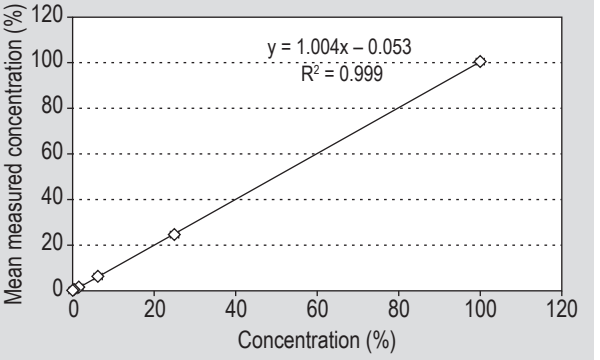

Figure 3. The correlation graph of mean measured concentration values and known concentration values of genetically modified (GM) soy with different GM contents.

4. Conclusions

Evaluation of method performance criteria, including all results, showed that the standard path in the detection method was suitable for GMOs present in the content of food, feed and seed. The methods, formulas and results provided guidance for the verification of qualitative and quantitative detection methods and the measurement of uncertainty based on single laboratory results for other GM events. On the other hand, GMO testing laboratories in Turkey need collaborative trials that explain how to estimate the analytical variability of qualitative and quantitative analytical results, immediately.

Acknowledgements

This study was supported by the scientific research foundation of Middle East Technical University (BAP-08-11-2012-007). We are thankful to our colleagues Pelin Kaya, Gökçen Zorbek, Erdem Boy, Işkın Engin who provided expertise that greatly assisted in the research. Also we are thankful to Prof. Dr Ayhan Temiz and Sean C. Lawrie (J.D.) for their careful reading of the manuscript.

References

Brooks, G., 2012. Economic impacts of the Biosafety Law and implementing regulations in Turkey on the Turkish importing and user sectors. PG Economics Ltd, London, UK.

European Network of GMO Laboratories (ENGL), 2008. Definition of minimum performance requirements for analytical methods of GMO testing. European Network of GMO Laboratories, Ispra, Italy.

European Commission (EC), 2008. Report on the validation of a DNA extraction method for soybean seeds. European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), Ispra, Italy.

European Commission (EC), 2014. Report on the validation of a DNA extraction method for soybean seeds. European Union, Institute for Health and Consumer Protection, Ispra, Italy.

Global Agricultural Information Network (GAIN), 2016. Agricultural biotechnology update. USDA foreign agricultural report global agricultural information network, report number: TR6026. USDA, Washington, DC, USA.

International Standard Organization (ISO), 2005a. ISO 21098 Foodstuffs-nucleic acid based methods of analysis of genetically modified organisms and derived products-information to be supplied and procedure for the addition of methods to ISO 21569, ISO 21570 or ISO 21571. ISO, Geneva, Switzerland.

International Standard Organization (ISO), 2005b. ISO 21571:2005 Foodstuffs-methods of analysis for the detection of genetically modified organisms and derived products-nucleic acid extraction. ISO, Geneva, Switzerland.

International Standard Organization (ISO), 2006. ISO 24276:2006 Foodstuffs-methods of analysis for the detection of genetically modified organisms and derived products-general requirements. ISO, Geneva, Switzerland.

Kamle, S. and Ali, S., 2013. Genetically modified crops: detection strategies and biosafety issues. Gene 522: 123-132.

Lim, H., Hwang, H.J., Kim, A.R., Cho, M.H., Ji, H., Kim, C.K., Ji, S.U., Cho, J.I., Park, S.C. and Lee, G.S., 2016. A simple, rapid and systematic method for the developed GM rice analysis. Plant Biotechnology Reports 10: 25-33.

Masip, G., Sabalza, M., Pérez-Massot, E., Banakar, R., Cebrian, D., Twyman, R.M., Capell, T., Albajes, R. and Christou, P., 2013. Paradoxical EU agricultural policies on genetically engineered crops. Trends in Plant Science 18: 312-324.

Mazzara, M., Munaro, B., Larcher, S., Grazioli, E., Delobel, C., Savini, C., Van den Eede, G., 2007. Event-specific method for the quantification of soybean line 40-3-2 using real-time PCR – validation report and protocol – report on the validation of a DNA extraction method for soybean seeds. EUR 23086 EN, Luxembourg, Luxembourg.

Monsanto, 2000. Updated molecular characterization and safety assessment of Roundup Ready soybean event 40-3-2. Monsanto Product Safety Center, St. Louise, MO, USA.

Pfaffl, M.W., Horgan, G.W. and Dempfle, L., 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research 30: e36.

Querci, M., Jermini, M. and Van den Eede, G., 2006. The analysis of food samples for the presence of genetically modified organisms. European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), Ispra, Italy.

Trapman, S., Burns, M., Broll, H., Macarthur, R., Wood, R. and Zel, J., 2009. Guidance document on measurement uncertainty for GMO testing laboratories. Institute for Reference Materials and Measurements, Luxembourg, Luxembourg.

Waiblinger, H.U. and Grohmann, L., 2014. Guidelines for validation of DNA extraction methods applied in subsequent PCR analysis of food and feed products for the presence of genetically modified material. Journal für Verbraucherschutz und Lebensmittelsicherheit 9: 183-190.