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Exploratory and confirmatory molecular approaches to determine genetically modified status in different crops

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Abstract

Background: One of the parameters required for the assessment of food and feed safety is detection and identification of genetically modified organisms. Legislation in some countries necessitates detection and quantification of modification in food and feed samples. Scientists have raised concern about safety of antibiotic resistance marker (ARM) genes used for transformation of crops intended for human and animal consumption. In the present work two molecular approaches have been adopted: one exploratory; for detection and quantification of ARM genes in tested plant samples and the other confirmatory; to determine the specificity/reliability of the obtained results.

Results: Results revealed that primers for neomycin phosphotransferase (*nptII*) and aminoglycoside 3″ adenyltransferase (*aadA*) were amplified in the majority of the 36 DNA screened samples. Melting curve analysis using hygromycin phosphotransferase (*aphIV*) gene as target sequence for the fluorescent-based detection approach was performed to ensure reliability and specificity of this procedure and to confirm results obtained by using conventional polymerase chain reaction (PCR). Quantitative RT-PCR results and validation analysis followed, revealed that all of the tested DNA samples were not violating the European legislation for GMOs labeling (0.9%).

Conclusions: The results fully demonstrated the reproducibility, sensitivity/specificity of the adopted approaches for detection and quantification of even traces of GMO contents. Applying measurement uncertainty (MU) procedures presented in this work will help decision makers to ensure compliance with International Legislation and Regulations. This in its turn will facilitate and enhance trading with countries having compelling labeling regulations.

Keywords: Genetically modified organisms, GM crops, PCR, Melting curve, RT-PCR, Measurement uncertainty

Background

The technology of genetically modified organisms (GMOs) is mainly directed toward increasing the yield of a particular crop by introducing resistance to herbicides, insect pests, and certain diseases or by improving storage, transport, and harvest characteristics (James 1999; Mathur et al. 2017). The genetic composition of a GMO is usually altered by insertion of a piece of DNA having a desired trait into the genome of a recipient organism. This process called transformation. A typical insert in a GMO is composed of a promoter to start signaling, the gene of interest, and a terminator to stop signaling. Several other elements are used in a gene construct; as antibiotic resistance marker genes to confirm the presence of genetic modification. The use of antibiotic resistance marker genes in the development of GMOs has raised concern worldwide about the safety of these genes in GM crops intended for human and animal consumption, which highlighted the increased demand for reliable and accurate analytical methods for detection and quantification of GM products (Mafra et al. 2008).

Legislation in some countries requires traceability, detection and quantification of GMOs to comply with labeling regulations for products that contain GMOs

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above a certain threshold (Cankar et al. 2008). Strict EU regulations necessitate detection and quantification of GM events in food and feed samples. Regulations also necessitate estimation of the measurement uncertainty that originates during analytical section of the detection procedure.

Monitoring of GMOs is considered an important procedure in assessing the level of safety in the release of GMOs into the environment. Several approaches have been developed to detect GM food or feed applying protein-based techniques, RNA- and DNA- based techniques (Salisu et al. 2017) to search for the inserted foreign protein or gene. One of the latest developed protein-based technique (Zenga et al. 2021) is the colloidal gold immune chromatographic strip (ICS) which is used for simultaneous detection of multiple transgenic proteins. The DNA-based approaches, on the other hand, are considered the methods of choice, DNA-based methods rely on the inherent stability of its double stranded structure and the possibility of multiplying it using the polymerase chain reaction (PCR) technology (Anklam et al. 2001).

This work employed three of the DNA-based molecular techniques; the conventional polymerase chain reaction (PCR), the fluorescent-based detection technique (Farrar and Wittwer 2017) using real time-PCR and a fluorogenic TaqMan probe specific for *nptII* for detection and quantification of GM-crops.

Conventional PCR (C-PCR) was applied first for screening plant crops and animal feed for the presence of genetic modifications using two pairs of primers for Cauliflower Mosaic Virus promoter*; P-35S* and *GT88*. It was also applied to investigate the presence of the antibiotic resistance marker genes; neomycin phosphotransferase *(nptII),* and aminoglycoside 3″ adenyl-transferase *(aadA)*.

The hygromycin phosphotransferase (*aphIV*) antibiotic resistance marker gene was also used as target sequence for the fluorescent-based detection assay. Melting curve analysis was performed to ensure accuracy and specificity of the procedures and results.

Quantitative evaluation of genetic modification was carried out using the real-time polymerase chain reaction (RT-PCR) with TaqMan probe specific for *nptII* gene (101 bp-target) in presence of a standard reference material (CRM from Fluka).

Measurement uncertainty (MU) values were estimated in order to judge whether the analytical results exceeded a threshold or not. The MU values were estimated following the Guidance Document on Measurement Uncertainty for GMO Testing Laboratories (Trapmann et al. 2009), and further reported along with the measurement results.

Methods

Thirty two plant crops and four animal feed samples were purchased from different plant seed and animal feed suppliers. Plant crops were packaged in tins where identification particulars were provided. These purchased plant crops were imported from nine different countries distributed in Europe, United States of America (USA), and Asia where were produced. None of the purchased plants or animal feed was labeled as genetically modified.

Sampling and DNA extraction

Homogenous samples were prepared by milling approximately 100–250 g of starting material in a grinding food machine. One gram of each sample was further grounded to fine powder by the help of liquid nitrogen. DNA was extracted in duplicate from all samples applying a modified CTAB-based method (Aboul-Maaty and Oraby 2019).

Qualitative detection for genetic modification

For screening purposes, we employed two molecular procedures for qualitative detection methods. The first based on the use of conventional PCR and the second was a Fluorescent-based detection approach using real time-PCR (Farrar and Wittwer 2017).

Conventional PCR approach (C-PCR)

Detection of the presence of genetic modification was performed using two pairs of primers to amplify two segments from *CaMV* promoter; *P-35S* (195 bp-target) and *GT88* (88 bp-target) using C-PCR. Two other pairs of primers (*nptII* and *aadA*) were synthesized to amplify segments from *nptII* gene (173 bp-target) and *aadA* gene (284 bp-target) respectively. Sequences, annealing temperatures and amplicon lengths of primers used during this study are presented in Table 1.

Amplifications were carried out in a total volume of 25 µl reaction mixture which contained 100 ng template DNA, 2 mM $MgCl_2$, 50mMKCl, 200 µM of each dNTP, 2.5 pmol of each primer and 2.5 units of taq DNA polymerase in a reaction buffer (75 mM Tris–HCl, pH 8.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, and 0.001% BSA). All Polymerase Chain Reactions were conducted at least in duplicates and accomplished in TM Thermal cycler (MJ Research PTC-100 thermocycler) programmed to perform an initial denaturation step of 95 °C for 2 min, followed by 40 cycles consisting of 30 s at 95 °C for denaturation, 45 s at annealing temperature for each primer (Table 1), and 30 s at 72 °C for extension. A final extension step of 7 min at 72 °C was performed. Following completion of the cycling reaction, 2 µl of a tracing dye (1 µg/ml ethidium bromide) was added to 10 µl of each reaction product and separated by 2% agarose gel

Table 1 List of primers employed for detection and quantification of GMOs, Primers positions[a] on the genomes, sequences, amplicon lengths and annealing temperatures

Primers	Positions on the genomes[a]	Sequences (5′–3′)	Amplicon length (bp)	Annealing temp. (°C)	References
P-35S	7190–7209	5′–GCTCCTACAAATGCCATCA–3′	195	57	Hemmer (1997)
	7364–7384	5′–GATAGTGGGATTGTGCGTCA–3′			
GT88	7117–7138	5′–TCCGGAAACCTCCTCGGATTCCAT–3′	88	54	Oraby et al. (2014)
	7183–7206	5′–GGCATTTGTAGGAGCCACCTTCCT–3′			
nptII	2382–2397	5′–GGATCTCCTGTCATCT–3′	173	50	Hemmer (1997)
	2539–2554	5′–GATCATCCTGATCGAC–3′			
nptII	2145–2167	5′–CTATGACTGGGCACAACAGACA–3′	101	60	Cited by Li et al. (2015)
	2225–2245	5′–CGGACAGGTCGGTCTTGACA–3′			
	Probe: 2172–2195	FAM–CTGCTCTGATGCCGCCGTGTTCCG–TAMRA			
aphIV	14,025–14,044	5′–CCGATTCCGGAAGTGCTTGA–3′	649	60.5	Designed for this work
	14,654–14,673	5′–CCCAAGCTGCATCATCGAAA–3′			
aadA	1188–1208	5′–CGC TAT GTT CTC TTG CTT TTG–3′	284	63	Hollingshead and Vapnek (1985)
	1451–1471	5′–TGA TTT GCT GGT TAC GGT GAC–3′			

[a] Primers positions relevant to: Cauliflower mosaic virus genome (accession no. emb|V00141.1|), *nptII* gene (accession no. AF080390.1*), aphIV* gene (accession no. KY080693.1) and *aadA* gene (accession no. MH973510.1)

electrophoresis and analyzed using SYNGENE Bio Imaging Gel Documentation System for the presence of a fluorescent band of the expected level for each primer.

Fluorescent-based detection approach

The fluorescent-based detection approach was performed using the real time-PCR (RT-PCR) in presence of 10 µl Maxima SYBR Green qPCR master mix and 1 µ of each forward and reverse DNA primers specially designed for the amplification of a segment of *aphIV* gene which was used as screening target for the presence of genetic modifications. Template DNA (150 ng of each sample) was added and the reaction volume was adjusted by DEPC water to a final volume of 20 µl. Amplification was started with initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Fluorescence measurements were obtained during the elongation step with SYBR Green1 dye. Additionally a final step for melting curve analysis was performed to ensure accuracy and specificity of the results.

Quantification of genetic modification in plant samples

Standard curve construction

Taq Man probe for *nptII* gene (101 bp-target) was synthesized by Applied Biosystem, UK (Table 1) to determine the percentage of *nptII* gene in selected plant seeds and feeds samples. Quantification of genetic modification in selected samples was determined in relation to a constructed standard curve from different dilutions (0.1, 0.5 and 5%) from the certified reference material (CRM; Fluka) using *nptII* gene (101 bp-target).

DNA templates were, prepared from the different dilutions of the certified reference standard material (CRM) following the standardized CTAB-based protocol (Aboul-Maaty and Oraby 2019). The standard curve was generated by blotting the log of the initial template GM percentages against the threshold cycle (Ct) generated for each concentration. Comparing the Ct values of the unknown samples to the standard curve allows the quantification of initial GM concentration percent for each sample. Real time-PCR was performed with the DNA of tested plant samples along with the DNA of different Fluka standard concentrations.

TaqMan RT-PCR conditions and amplification

Reaction mixture was prepared in 12.5 µl master mix (TaqMan Universal PCR Master Mix, ABI), 300 nM of each primer, 200 µM probe and 100 ng DNA. The final volume (25 µl) was adjusted by nuclease-free water. The quantitative real–time PCR analysis was carried out in an RT-PCR Cycler-Rotor-Gene Q 2 Plex-with 2 channels (QIAGEN) and data analyzed by the software version Rotor-Gene 2.0.2.4. RT-PCR amplification profile started by a hold step for 15 min at 95 °C followed by 40 repeats of 15 s at 94 °C and 60 s at 60 °C.

Estimation of measurement uncertainty relevant to GMO quantification

Data obtained from quantification of genetic modification in plant samples using TaqMan probe RT-PCR

were used for estimation of Measurement Uncertainty involved in the GMO analysis following the Guidance Document on Measurement Uncertainty for GMO Testing Laboratories (Trapmann et al. 2009). This approach also evaluates the parameters involved in the analysis that may cause uncertainty about the reproducibility of the results.

Results

Conventional polymerase chain reaction (PCR) approach

Results indicated that four of the collected plant samples gave no amplification with all investigated primers. These four plant samples are: yellow maize (Benicia variety from Ireland), and samples for Lupine, *Vicia faba*, and wheat from Egypt.

According to results in Table 2 primers specific for *35S* promoter (195 bp-target) and *GT88* were amplified in most of the screened plant samples (Figs. 1 and 2 respectively). The percentage of the presence of the two components (*P-35S* and *GT88*) of *CaMVP35S* promoter together in the same samples was 80.5% of the screened samples. On the other hand, the *GT88* (88 bp-target) fragment was separately identified in two samples; soybean (20) and golden rice (30). Primers for the *nptII* (173 bp-target) and *aadA* (284 bp-target) were amplified in 72.2% and 61.1% respectively of the DNA of the investigated samples (Table 2).

Collected plant crop and animal feed samples were also screened for the presence of antibiotic resistance marker genes; *nptII* gene, and *aadA* gene. Primers for the *nptII* (173 bp-target) were amplified in 71.87% of the collected plant samples and in 75% of the collected animal diets (Fig. 3).

Fluorescent-based detection approach for investigating the presence of *aphIV* gene qualitatively using real time-PCR

Figure 4 represents amplification plot (panel A) and melting curve (panel B) obtained by the fluorescent-based detection approach with SYBR Green I dye for the selected DNA samples. Analysis of the targets containing the third antibiotic resistance marker gene (*aphIV* gene) are listed in Table 3. The gathered information of the dissociation of double-stranded DNA amplicon of tested samples during heating (Fig. 4 panel B) showed that the peak detection of melting temperatures (T_m) ranged from 81.5 to 82.5 °C in all tested samples (Table 3) denoting positive amplification of the *aphIV* gene primers.

Quantitative detection of *nptII* gene in GM-crops applying TaqMan probe technology

The constructed standard curve (Fig. 5) from Fluka CRM dilutions (0.5, 2 and 5) using specific Taq Man Probe for *nptII* gene (101 bp target) demonstrated that the correlation co-efficient (R^2) value was equal to 0.999 and the reaction efficiency was equal to 92%, whereas the slope was −3.526. The limit of quantification (LOQ) was 0.49% as presented in Table 4.

The exact percent of the target sequence in some of the tested samples with unknown concentrations were further quantified against the constructed Fluka standard curve and presented in Table 5. The calculated mean concentration percent in five of the tested samples (6, 9, 13, 19 and 35) recorded higher values (1.088, 3.680, 1.92, 1.437, 2.95 respectively) than that of the permissible GM% (0.9%).

Estimation of measurement uncertainty parameters involved in the GMO quantification.

In order to evaluate the performance of the method employed in the present work (Table 5); different uncertainty parameters involved in the GMO quantification were further estimated.

The absolute difference ($\Delta_m = |C_m - C_{CRM}|$) between mean measured results and certified value ($C_{CRM} = 0.9$) was compared with the expanded uncertainty ($U_\Delta = k \cdot u_\Delta$) of the difference between our results and certified value. In the present work the calculated absolute difference (Δ_m) of each of the tested samples was smaller than the corresponding U_Δ (expanded uncertainty) of the same sample. These results denoted that there was no significant difference between the measurement result and the certified value. Based on that the difference between the reported GM concentrations and the expanded uncertainty (U_Δ) value for all samples were calculated (Table 5). Results indicated that none of the tested plants DNA samples exceeded the permissible concentration threshold (0.9%).

Discussion

During this work we adopted two approaches: one exploratory; to investigate the presence of genetic modification in tested plant samples and the other confirmatory to determine the specificity and reliability of the obtained results.

For the purpose of screening GM crops the conventional PCR assay was employed. The universal primers for Cauliflower mosaic virus promoter (*P-35S*; 195 bp) were successfully amplified in most of the tested samples (Table 2). Generally, fragments of the frequently used promoter or terminator are used for the detection of genetically modified plants (Duijn et al. 1999; Kok et al. 2000; Oraby et al. 2005). Twenty four different regions on the *CaMV* Promoter were previously reported (Wu et al. 2014) as detection methods for GMOs. Primers for *GT88* segment (Oraby et al. 2014) used in the present work is

Table 2 Detected genetic modifications in some of the collected plant samples using primers for *P–35S* (195 bp), *GT 88* (88 bp), *nptII* (173 bp) and *aadA1* (284 bp)

Code number	Samples	Genus species	P-35S (195 bp)	GT88 (88 bp)	NPTII (173 bp)	aadA (284 bp)
1	Yellow maize (Benicia)	Zea mays	−	−	−	−
2	Yellow corn	Zea mays	+	+	+	+
3	Potato 1	*Solanum tuberosum*	+	+	+	+
4	Potato 2	*Solanum tuberosum*	+	+	+	+
5	Potato 3	*Solanum tuberosum*	+	+	+	−
6	Potato 4	*Solanum tuberosum*	+	+	+	+
7	Potato 5	*Solanum tuberosum*	+	+	+	+
8	Potato 6	*Solanum tuberosum*	+	+	+	+
9	Water melon	*Citrullus lanatus*	+	+	+	+
10	Tomato	*Solanum lycopersicum*	+	+	+	+
11	Tomato	*Solanum lycopersicum*	+	+	+	−
12	Melon	*Cucumis melo*	+	+	+	+
13	Squash	*Cucurbitales maxima*	+	+	+	−
14	Cucumber	Cucumis sativus	+	+	+	−
15	Cucumber	Cucumis sativus	+	+	+	+
16	Cucumber	Cucumis sativus	+	+	−	−
17	Lupine	Lupinus Lupinus	−	−	−	−
18	Chick-pea	*Cicer arientinium*	+	+	−	+
19	French bean	Phaseolus vulgaris	+	+	−	+
20	Soybean	Vicia faba	−	+	+	−
21	Faba	*Lens culinaris*	−	−	−	−
22	Lentil	*Glycine max*	+	+	+	+
23	Sweet pepper	*Capsicum annuum*	+	+	+	+
24	Sweet pepper	*Capsicum annuum*	+	+	+	+
25	Cabbage	*Brassica oleracea*	+	+	+	−
26	Berenjena	*Solanum melongena*	+	+	+	−
27	Berenjena romy	*Solanum melongena*	+	+	+	−
28	Wheat (Romania)	*Triticum aestivum*	+	+	+	+
29	Wheat	*Triticum aestivum*	−	−	−	−
30	Golden rice	*Oryza sativa*	−	+	−	−
31	Thai rice	*Oryza sativa*	+	+	+	+
32	Cotton	*Gossypium arboreum*	+	+	−	+
33	Animal diet1 B (Cairo)		+	+	+	+
34	Animal diet 2 R (FMx)		+	+	−	+
35	Animal diet 3 (rodent)		+	+	+	+
36	Animal diet 4 B (FMX)		+	+	+	+

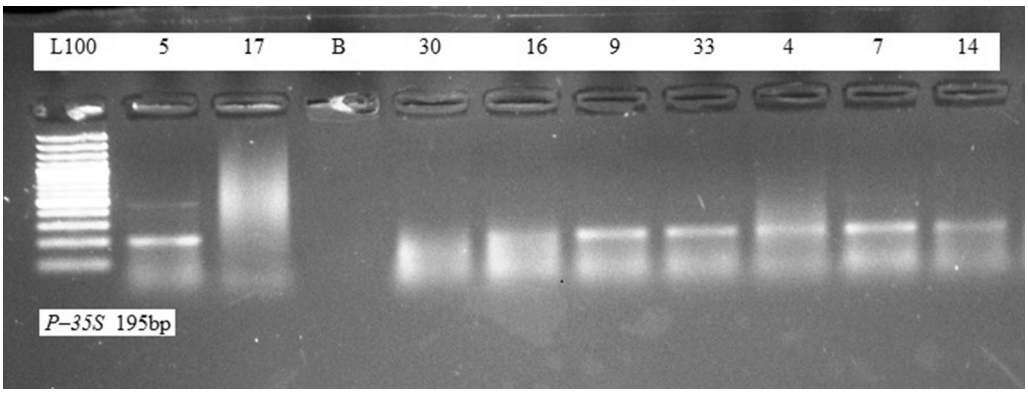

Fig. 1 Selected PCR amplification products of *P-35S* (195 bp) in some of the collected plant and animal feed samples**.** B is blank

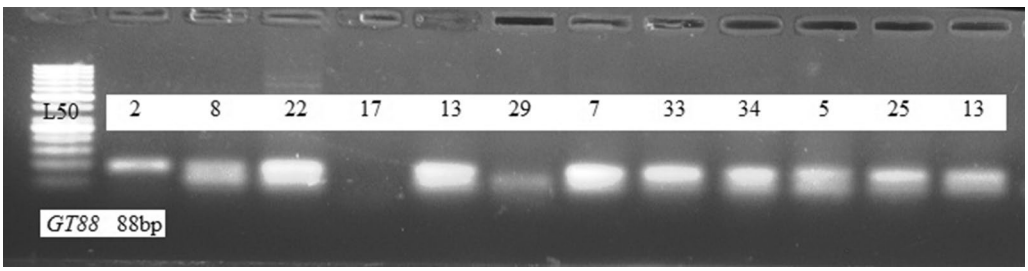

Fig. 2 Selected PCR amplification products of *GT88* (88 bp) in some of the collected plant samples

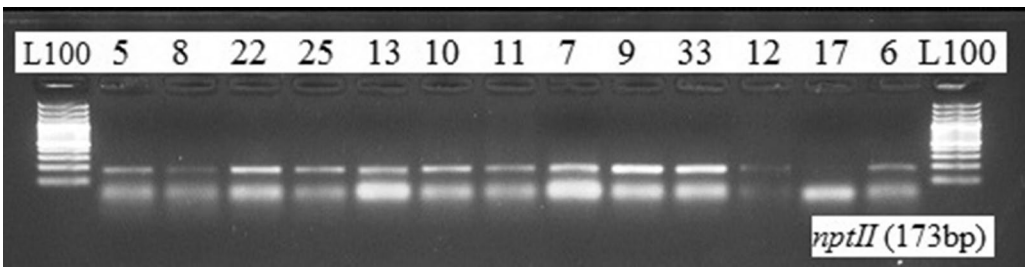

Fig. 3 Selected PCR amplification products of *nptII* (173 bp) in some of the collected plant samples. Sample 33 is for one of the tested animal feed

targeting a new region (7117–7206) on the *CaMV P-35S* promoter (accession no. emb|V00141.1|). This new set of primers (*GT88*) was amplified in 86.1% of the collected samples, whereas *P-35S* (195 bp) was amplified in 83.3%. Amplification results of *GT88*, in the present work not only supported the presence of *CaMV-35S* promoter in these samples, but also suggested the use of these primers as an additional method for screening GMOs since it is targeting a new region in the *CaMV-35S* promoter.

The conventional PCR assay was also applied for exploring the presence of fragments from antibiotic resistance marker genes; *nptII* (173 bp) and *aadA* (284 bp) in the collected plant samples. Primers for *nptII* and *aadA* were successfully amplified in 72.2% and 61.1% respectively of the collected samples. Fragments from both genes were detected together in 50% of the screened samples.

GM plants usually contain bacterial antibiotic resistance (AR) genes which are used as selectable marker genes in the early laboratory stages during their development. The bacterial *aadA* gene, coding for aminoglycoside 3″ adenyl transferase, is under the control by its own bacterial promote (Miki and McHugh 2004) which renders it inactive in plants thus it is not expressed in

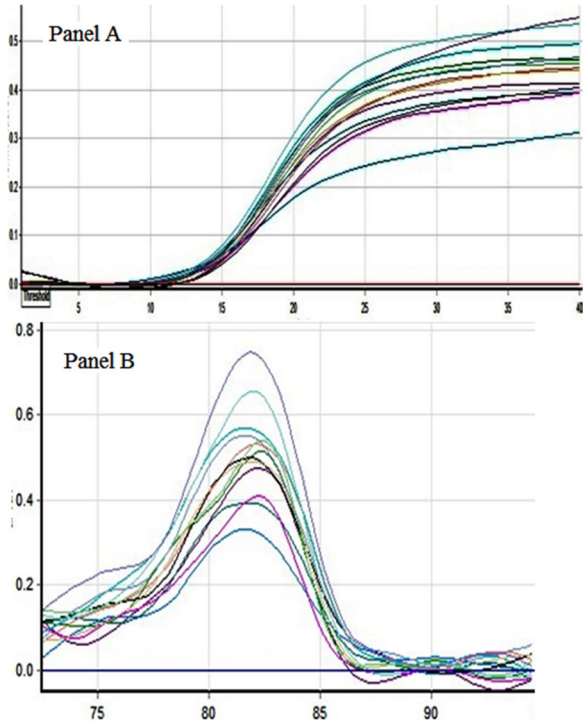

Fig. 4 Linear amplification plots (panels **A**) and melting curves (panel **B**) for *aphIV* gene in selected samples. **A** The cycle number is plotted on the *X*-axis versus the measured fluorescence increase on the *Y*-axis. **B** The temperature (°C) is plotted on the *X*-axis versus the measured fluorescence variation (d*F*/d*T*) on the *Y*-axis

Table 3 Melt curve analysis: rate of changes of fluorescence due to amplicon dissociation of *aphIV* in tested samples and peak detection range data

Plant samples		Melting temperatures (T_m)
Code	Name	peak detection range data (°C)
Level 4	Fluka CRM 1%	82.2
12	Melon	82.0
14	Cucumber/Hybrid Assel/France	82.0
16	Cucumber/Hybrid beit alpha/USA	82.3
20	Soybean/Egypt	82.0
24	Sweet pepper/Moaz/USA	82.2
25	Cabbage	81.5
28	Wheat/Romania	81.8
30	Golden rice/Thailand	81.7
31	Jasmine rice/Thailand	81.7
32	Cotton/Egypt	82.0
35	Animal diet/NRC/Egypt	81.5
36	Animal diet 4B (FMX)	82.5

GM plants. The *nptII gene* on the other hand has mostly been used as selectable marker genes (Vidhya et al. 2012) under the control of plant promoter in transgenic plants. For more efficient selection methods, *nptII* in some cases is used in combination with aminoglycoside phosphotransferase gene rather than using it alone (Kumar et al. 2004; Tabatabaei et al. 2017). This was confirmed in our work where the presence of both genes (*nptII* and *aphIV*) was detected in some of the investigated samples when applying the conventional-PCR and the fluorescent-based detection approaches.

Some authors (Miki and McHugh 2004) reported that such markers are routinely eliminated prior to plant transformation. They claimed that markers conferring resistance to hygromycin or other antibiotics have been used in plant research (Day 2003), but do not appear in GM-plants. Our results showed evidence for the presence of these ARM genes in most of the screened plant and diet samples. Adugna and Mesfin (2008) also used nptII gene as screening element for detection and quantification of GM crops. In addition, presence of nptII was reported in transgenic pigeon pea plants (Surekha et al. 2005) and transgenic cotton samples (Vidhya et al. 2012). It has also been reported that *nptII* gene was used for the production of most citrus transgenic plants (Ballester et al. 2008).

To overcome the limitation of detection of GMOs using conventional PCR the fluorescent-based detection approach using a DNA binding dye (SYBR green 1) in presence of *aphIV* primers as target sequence was also implemented as a confirmatory approach for detection of GMOs. This approach was followed by the melt curve analysis to estimate the specificity of the amplified products based on their melting characteristics of the double stranded DNA (dsDNA) during heating (Farrar and Wittwer 2017). Due to its dependence upon the length of the product and the type of its nucleotides component this assay allowed for the differentiation between the target specific amplicon and any non-specific amplicons (Nolan et al. 2013). The gathered information presented in Table 3 showed that primers for *aphIV* were positively amplified in all selected samples. These results reflect the importance of applying this assay as a complementary approach to the conventional PCR for the detection of GM plants since, screening results using the conventional PCR approach showed that some of the plant samples (14, 16, 20, 25, 30 and 32) were found to be negative to one or more of the other investigated primers (Table 2). These results also supported the suggestion of Anklam et al. (2001) that the absence of one or two of the screening elements in tested plant samples do not signify that these samples are not modified, it rather

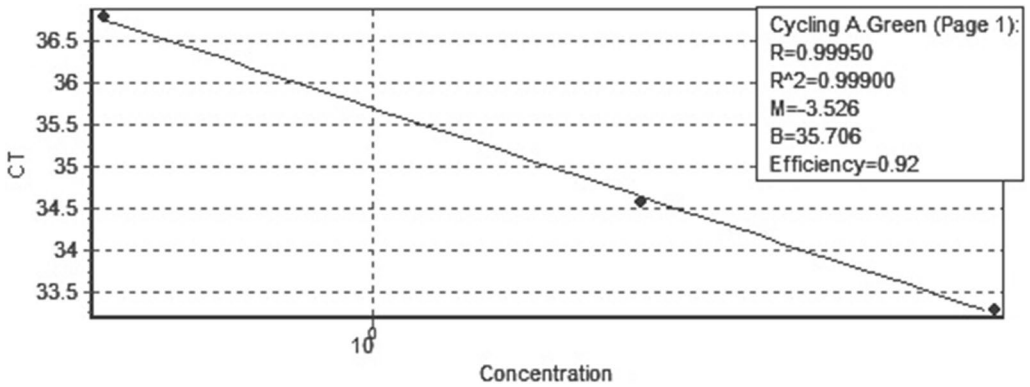

Fig. 5 Standard curve generated from Fluka CRM dilutions (0.5, 2, 5) using Taq Man Probe

Table 4 Calculated initial concentration percent for the standard curve constructed from different dilutions from the Fluka CRM

Code	Type	Ct	Given conc (%)	Calc conc (%)
1	Standard	37.07	0.500000	0.490130
2	Standard	34.81	2.000000	2.102754
3	Standard	33.51	5.000000	4.851434

recommends using more than one primer for screening plant samples for the presence of genetic modification. As it is well known that no one method can detect all commercially available transgenic events (Wu et al. 2014) due to the different methods used for the construction of plant transformation. In the present work using different DNA-based methods for screening the

Table 5 Measurement Uncertainty using the value of within-laboratory measurement variation

Sample number	Calculated concentration %			Sr	$u_m = \frac{Sr}{\sqrt{n}}$	$u_\Delta = \sqrt{u_m^2 + u_{CRM}^2}$	$U_\Delta = k \cdot u_\Delta$	$\Delta_m = \lvert C_m - C_{CRM} \rvert$	Estimated enforcement level[a]
	R (1)	*R* (2)	Mean% (C_m)						$C_m - U_\Delta$
3	0.2385	0.1545	0.1965	0.059397	0.0424	1.5255	3.051	0.7035	– 2.855
6	1.2499	0.9262	1.08805	0.22889	0.163	1.533	3.067	0.18805	– 1.979
7	0.1492	0.1959	0.17255	0.033022	0.024	1.525	3.05	0.72745	– 2.877
8	0.1477	0.1275	0.1376	0.014284	0.01	1.525	3.05	0.7624	– 2.912
9	3.1444	4.2174	3.6809	0.758726	0.542	1.618	3.237	2.789	0.444
12	0.0807	0.0888	0.08475	0.005728	0.004	1.525	3.049	0.81525	– 2.964
13	1.937	1.903	1.92	0.024042	0.017	1.525	3.05	1.02	– 1.13
16	0.2349	0.6506	0.44275	0.293944	0.20996	1.539	3.078	0.2494	– 2.635
19	1.481	1.393	1.437	0.062225	0.045	1.526	3.051	0.537	– 1.614
22	0.2304	0.2469	0.23865	0.011667	0.008	1.525	3.05	0.66135	– 2.811
23	0.4035	0.2364	0.31995	0.118158	0.008	1.527	3.055	0.58005	– 2.735
24	0.34	0.2724	0.3062	0.047800	0.03414	1.525	3.05	0.5938	– 2.744
25	0.3579	0.3737	0.3658	0.011172	0.008	1.525	3.05	0.5342	– 2.684
27	0.1533	0.1296	0.14145	0.016758	0.012	1.525	3.05	0.7704	– 2.909
30	0.1985	0.2271	0.2128	0.020223	0.0144	1.535	3.069	0.6872	– 2.856
31	0.2244	0.9168	0.5706	0.489601	0.3497	1.565	3.129	0.3294	– 2.558
32	0.7136	0.9494	0.8315	0.166736	0.119	1.529	3.059	0.0685	– 2.228
35	2.4518	3.45	2.9509	0.705834	0.50417	1.606	3.212	2.0509	– 0.261

[a] Maximum concentration stipulated in EU is 0.9%, R (1) is reaction 1, R (2) is reaction 2. u_m; uncertainty of measurement results, *Sr*; standard deviation of repeatability, *n*; number of independent measurement results, u_Δ; combined uncertainty of results and certified value, U_{CRM}^2 is uncertainty of the certified value. *k*; coverage factor equal to 2

investigated plant samples also, confirmed the presence of genetic modifications in these samples.

To guarantee traceability of the GMOs, several strategies have been developed to detect GMOs in food/feed samples by using different technologies. In most cases, GMO screening approaches also apply quantitative methods for detecting the presence of GM material in food and feed samples (Barbau-Piednoir et al. 2014). Many countries have imposed different biosafety laws (De Jong 2010) and GMOs labeling policies with a threshold of tolerance varying between 0 and 5% which are controlled by their competent authorities (Kamle and Ali 2013).

In the present work the standard curve approach using the Fluka certified reference material (Fluka CRM) was chosen for GMO quantification. This approach is based on absolute quantification rather than relative quantification approach (Weighardt et al. 2004) which based on the use of reference gene for normalization.

A series of parameters has to be considered to validate and verify the accuracy and the performance characteristics of the quantification method applied. One of these parameters is the squared correlation coefficient (R^2) of the constructed standard curve. For a well-optimized reaction the R^2 value should be close to 1 and greater than 0.98 (Nolan et al. 2013). In our case R^2 value was 0.999. The dynamic range of concentrations; over which the method performed in a linear manner, is also another important performance characteristic parameter. In the present work the dynamic range of the standard curve showed a linear increase from 0.49 to 4.87%. It is worthy of note here that the dynamic range of concentrations should not exceed five times the permissible concentration (0.9%) of genetic modification (Del Gaudio et al. 2012).

Figure 5 showed that 92% reaction efficiency of the constructed standard curve indicating high efficiency and repeatability of the method employed as predicted from the line of best fit (slope) for the standard curve (− 3.526) in the present work. It has been reported that when a tenfold serial dilution is performed, the amplification plots for each dilution should be 3.3 cycles apart. In our case (Table 4) the amplification plots ranged from 2.26 to 3.56 cycles (Ct) apart. This difference in assay performance could be a result of using different dilutions (0.5, 2, and 5%) for the construction of standard curve in the present work or as suggested previously by Nolan et al. (2013), that it could even be related to different syntheses of the primer pair. Others (Morisset et al. 2009) reported that this difference could also be due to mismatches in the inserted sequences that have arisen during plant crossing.

Further and according to trueness the method applied here showed no bias (Table 5), since the absolute difference (Δ_m) between mean measured values (C_m) and certified value (C_{CRM}) were smaller than expanded uncertainty (U_Δ) of difference between result and certified value (Trapmann et al. 2014).

Additionally, Table 5 presented along with the calculation of measurement uncertainty (MU), the estimated enforcement level for each plant samples to ensure compliance with the EU 0.9% legislation. The calculated difference between the reported GM concentrations (C_m) and the expanded uncertainty (U_Δ) value for all samples did not exceed the permissible concentration threshold (0.9%). Since it is not easy to avoid contamination during storage or transport of GM crops, these results indicated that the tested plant crops were considered not violating the European legislation for GMOs labeling. It also reflects the sensitivity of this approach to detect even traces of GM content in DNA of plant samples.

The validation study necessitates covering all the steps in the method to ensure evaluation of all parameters that may influence the result. One of the important parameters related to method validation as proposed by Holest-Jensen and Berdal (2004) is validation of DNA extraction procedures from different sample matrixes. It is well known that isolation and purification of DNA is a crucial step in DNA molecular techniques used in plant studies for the assessment of food safety (Sönmezoğlu and Keskin 2015), especially with the increase of the global cultivation area of genetically modified (GM) crops. For reliable results extraction of the DNA from all tested plant samples was performed, in the present work applying the same DNA extraction protocol to avoid any possible different composition or substances that may affect the efficiency of the PCR assays. This protocol is a modified CTAB-based method specially developed in our laboratory (Aboul-Maaty and Oraby 2019) for isolation of high quality and purity DNA from different plant orders.

Conclusions

The present work was mainly, conducted in the context of building capacities for detection and quantification of genetically modified (GM) crops applying exploratory and confirmatory molecular approaches. The results fully demonstrated the reproducibility, sensitivity / specificity of the adopted approaches for detection and quantification of even traces of GMO contents.

Applying measurement uncertainty (MU) procedures presented in this work will help decision makers to ensure compliance with International Legislation and Regulations. This in its turn will facilitate and enhance trading with countries having compelling labeling regulations.

Abbreviations
ARM: Antibiotic resistance marker; nptII: Neomycin phosphotransferase; DNA: Deoxyribonucleic Acid; aadA: Aminoglycoside 3″ adenyl-transferase; aphIV: Hygromycin phosphotransferase; PCR: Polymerase chain reaction; GMOs: Genetically modified organisms; RT-PCR: Reverse transcription polymerase chain reaction; MU: Measurement uncertainty; C-PCR: Conventional polymerase chain reaction; P-35S: Cauliflower mosaic virus promoter; CRM: Certified reference material; dNTP: Deoxyribonucleotide triphosphate; CTAB: Cetyltrimethylammonium bromide; Ct: Threshold cycle; SYBR green: Synergy brands; T_m: Melting temperatures; LOQ: Limit of quantification; U_Δ: Expanded uncertainty; Δ_m: Absolute difference; C_m: Mean measured values; C_{CRM}: Certified value.

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Authors' contributions
HO made substantial contributions to conception, planning of the work, analysis and interpretation of results. Also involved in, drafting the manuscript and revising it critically for important intellectual content; as well as given final approval of the version to be published. NA-M made substantial contributions to conception and design of the work, involved in conducting the practical section of the work, and the measurement uncertainty statistical analysis. Also involved in, drafting the manuscript; as well as given final approval of the version to be published. HA-S involved in conducting the practical section of the work. Partially involved in, conducting the measurement uncertainty statistical analysis; as well as given final approval of the version to be published. Each author has participated sufficiently in the work to take public responsibility for appropriate portions of the content; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The authors have participated sufficiently in this work. All authors have read and approved the final manuscript.

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Availability of data and materials
Authors declare that all data generated or analyzed during this study are included in this article.

Declarations

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Not applicable.

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