DNA extraction techniques compared for accurate detection of genetically modified organisms (GMOs) in maize food and feed products

Aydin Turkec · Hande Kazan · Burçin Karacanli ·
Stuart J. Lucas

Revised: 1 June 2014 / Accepted: 1 September 2014 / Published online: 17 September 2014
© Association of Food Scientists & Technologists (India) 2014

Abstract In this paper, DNA extraction methods have been evaluated to detect the presence of genetically modified organisms (GMOs) in maize food and feed products commercialised in Turkey. All the extraction methods tested performed well for the majority of maize foods and feed products analysed. However, the highest DNA content was achieved by the Wizard, Genespin or the CTAB method, all of which produced optimal DNA yield and purity for different maize food and feed products. The samples were then screened for the presence of GM elements, along with certified reference materials. Of the food and feed samples, 8 % tested positive for the presence of one GM element (NOS terminator), of which half (4 % of the total) also contained a second element (the Cauliflower Mosaic Virus 35S promoter). The results obtained herein clearly demonstrate the presence of GM maize in the Turkish market, and that the Foodproof GMO Screening Kit provides reliable screening of maize food and feed products.

Keywords Food · Feed · GM maize · DNA extraction · Multiplex real-time PCR screening

Introduction

The rapid expansion of recombinant DNA techniques has led to more genetically modified (GM) crops and/or their products being released onto the market worldwide over recent decades (Kluga et al. 2012). Consumer awareness of food safety issues and legislation regarding the rights of the consumer has resulted in an increasing need to enable safety assessments and enforce labeling requirements by verifying any labeling statements (Mafra et al. 2008). The European Union (EU) regulate the requirements set for genetically modified organism (GMO) usage in food and feed products, and require the mandatory labeling of food products containing more than 0.9 % authorized GMO and more than 0.5 % unauthorized GMO material for the consumers' information (European Commission Regulation (EC) 2003a, 2003b). Monitoring the presence of genetically modified organisms in the food and feed industry has become a challenge; as a result several available analytical methods have been developed for GMO detection (Jasper et al. 2009; Querci et al. 2009; Kluga et al. 2012). The polymerase chain reaction (PCR) including real-time PCR (RTi-PCR) is the most commonly used of the GMO detection approaches (Miraglia et al. 2004). Since a prerequisite to GMO detection by PCR is the ability to obtain high quality extracted DNA, the choice of DNA extraction method is of critical importance for successful application of these methods (Tung-Nguyen et al. 2009; Smith et al. 2007). The different extraction methods can result in DNA showing different levels of purity and yield in different types of food matrices and feed, necessitating the validation of DNA extraction methods on a case-by-case basis (Tung-Nguyen et al. 2009; Gryson 2010; Branquinho et al. 2010).

Maize is an important staple food ingredient of which a growing percentage of the world's production is derived from GM varieties (Meyer 1999; Kluga et al. 2012; James 2012). The presence of GM maize DNA in maize-containing products sold in the EU and Turkey is highly probable, since

A. Turkec (✉)

Uludag University Plant and Animal Production Department,
Mustafa Kemalpaşa Vocational School, 16500 Bursa, Turkey
e-mail: aturkec@uludag.edu.tr

H. Kazan · B. Karacanli

Elips Health Products Ltd, Atatürk mh. Namık Kemal cd no: 17, Tan Plaza, Ataşehir, Istanbul, Turkey

S. J. Lucas

Sabancı University Nanotechnology Research and Application
Centre, Orhanlı, 34956, Tuzla, Istanbul, Turkey

several GM maize varieties have been authorized for food and feed use in the EU (Kluga et al. 2012; Arun et al. 2013). In Turkey, 16 different GM maize events have been approved for use in animal feeds, but not foodstuffs (Gain report 2013).

The aims of our study were twofold: firstly, the verification of DNA extraction methods to ascertain which is most appropriate to yield good and high quality maize DNA from a variety of products, and secondly screening for GMO DNA in maize-containing food and feed products commercially available in the Turkish market. Four commercial DNA extraction kits, Foodproof, Genespin, Wizard, and DNeasy, and the CTAB (Cetyltrimethylammonium bromide) DNA extraction method were evaluated. Their efficiency was compared in terms of DNA yield and purity, and suitability for amplification through multiplex real-time PCR with the Foodproof GMO Screening Kit.

Material and methods

Samples and reference materials Forty-nine maize foodstuffs (flour, starch, bread, flakes, chips, maize-based biscuit, diet breakfast cereal, canned maize seed and feed samples, imported or locally produced with imported ingredients, were purchased from random local stores in Turkey. The samples, obtained from different suppliers and/or brands, were grouped in order to have reliable and wider results on each type of food and feed products (Mafra et al. 2008). The certified reference materials (CRMs) consisting of maize powder from Bt11 (ERM-BF412), Bt176 (ERM-BF411), GA21 (ERM-BF414), MON810 (ERM-BF413k), TC1507 (ERM-BF418), and NK603 (ERM-BF415) GM plants were obtained from Sigma-Aldrich and used as controls.

DNA extraction methods The DNA extraction methods were chosen from those previously reported to be successful for food and feed products (Mafra et al. 2008; Peano et al. 2004; Smith et al. 2007; Jasper et al. 2009; Wang et al. 2012). Total DNA was isolated with four commercial kits: Foodproof GMO Sample Preparation (Biotecon Diagnostics GmbH, Potsdam, Germany), DNeasy Plant Mini (Qiagen, Valencia, CA), Wizard (Promega, Madison, WI), and Genespin (GeneScan, Freiburg, Germany) as described by the manufacturers' instructions, and by the CTAB (Cetyltrimethylammonium bromide) method with some modifications, specifically adding RNase A (10 mg/mL) in the DNA precipitation step (Mafra et al. 2008). 100 mg samples were used for all extractions with exception of the Genespin method (200 mg samples) as recommended in the producer's manual (Cankar et al. 2006). The DNA yield and purity were evaluated using the NanoDrop 1000 UV/Vis spectrophotometer (Thermo Scientific) and 1 % agarose gel electrophoresis. All samples were extracted in triplicate to assay

reproducibility of the methods and extracted DNA was stored at -20°C for subsequent steps.

The evaluation of DNA extraction methods for real-time PCR All extracted samples were screened by real-time PCR using a plant-specific control gene provided in the Foodproof GMO Screening Kit (Biotecon Diagnostics GmbH, Potsdam, Germany) as a reference and control for DNA extraction efficiency (Fandke 2002). In addition, the PCR efficiency and the linearity of the calibration curves obtained for this reference by each extraction method were evaluated by the amplification of serially diluted extracts of CRM (5 % Bt11 DNA), which is an unprocessed sample with defined particle size (Cankar et al. 2006). The extracted DNA was first diluted to the concentration used in the reactions ($40\text{ ng}\cdot\mu\text{L}^{-1}$) (Kluga et al. 2012). From this sample, a serial fourfold dilution series was prepared (from 1:4 to 1:256) in triplicate. Ct (threshold cycle) values measured for the diluted samples were plotted against the logarithm of the dilution factor. The efficiency of the PCR reaction was then calculated by the formula: $E = ([10^{(-1/\text{slope})}] - 1) \times 100\%$ (Bustin and Nolan 2004).

GMO screening A multiplex real-time PCR screening kit, the Foodproof GMO Screening Kit (Biotecon Diagnostics GmbH, Potsdam, Germany) was used to detect plant-derived DNA and GMO target sequences (P-35S, T-NOS, bar gene and FMV P-35S) in maize foodstuffs and feeds (Dorries et al. 2010). PCR reactions were set up according to the manufacturer's instructions, mixing 1 μL enzyme solution and 1 μL dye solution (35S/NOS/bar/FMV system) or Internal Amplification Control (Plant Gene system), with 18 μL of the appropriate Master Mix. To each 20 μL PCR reaction mix was added 5 μL PCR grade water (negative control), control DNA, or sample DNA for a final volume of 25 μL . Real-time PCR was carried out on the LightCycler 480 Instrument (Roche Diagnostics GmbH, Mannheim, Germany). The reaction was incubated at 37°C for 4 min and 95°C for 10 min followed by 50 cycles at 95°C for 5 s and 60°C for 60s. Negative and positive control DNA provided with the kit were analyzed and compared with the corresponding results, and their Ct values used to determine the presence or absence of 35S/NOS/bar/FMV and plant genes in food and feed samples. All PCR reactions were performed in six replicates. For control and validation, the reference materials were included in all assays.

Statistical analyses All statistical analyses were performed using ANOVA models in SSPS 20.0 (IBM SSPS statistics, USA). When method, matrix effects or interactions were significant, Duncan's multiple range test was applied to compare the significant differences at 95 % probability (Wang et al. 2012). To estimate differences in amplifiability of serial dilutions of extracted DNA, two-way ANOVA ($\alpha=0.05$) was

used to compare the calculated real-time PCR efficiencies, slopes and correlation coefficients (R^2) (Zel et al. 2008). The comparative analysis of Ct values was performed using the Shapiro-Wilk distribution and the Levene test to check for homoscedasticity of variances (Mafra et al. 2008).

Results and discussion

Assessment of the quality of DNA extraction methods for maize food and feed products Four commercial kits and the CTAB extraction method were evaluated for different kinds of maize foodstuffs and feeds, in regard to DNA yield and purity, and the efficiency of PCR amplification from the purified DNA. All samples extracted were evaluated both spectrophotometrically (Nanodrop) and by 1 % agarose gel electrophoresis. The analysis of variance showed that the method, matrix and method-matrix interaction all contained statistically significant differences in DNA yield and purity as well as PCR efficiency at the 95 % confidence level ($p < 0.0001$) (Table 1). Therefore the analysis was carried out separately for each of the matrices, in order to determine the performance of the extraction methods for each matrix.

The comparison of five DNA extraction methods in this study demonstrated that the DNA extraction method can produce significantly different DNA yields and purity for different maize foodstuffs and feeds. On the other hand, irrespective of the extraction method used, the presence of a smear when extracted DNA was loaded on to an agarose gel indicated a certain level of DNA fragmentation (data not shown). (Kluga et al. 2012). According to the test results, although DNA yields differed generally with the type of extraction method, the poor values of purity obtained for intermediate or highly

processed maize products such as maize-based biscuit, maize bread and maize starch could be explained by the intensity of the industrial processing (Di Bernardo et al. 2007). The methods with purity ratios above 1.9 may indicate some contamination of the extracted DNA with ribonucleic acid (RNA) (Jasper et al. 2009).

Based on the statistical analysis (Table 2), the CTAB method was the most favorable method for extracting high amounts of DNA from maize bread, chips, maize-based biscuit, diet breakfast cereal, canned maize, and maize feed as well as maize seed, compared to the commercial kits. These observations were in accordance with previous studies, in which the CTAB method was generally considered the best for highly processed food matrices (Gryson et al. 2004; Cankar et al. 2006; Pironi et al. 2010), raw soybean, raw maize and animal feed (Tung-Nguyen et al. 2009). The CTAB method is also considered a method of choice for the extraction of DNA from leaves, seeds, and processed food/feed (ISO 2005). Similarly, Di Bernardo et al. (2007) verified the optimal performance of CTAB method as well as Epicentre method in majority of foodstuffs including maize bread, when compared to other commercial kits. The purity ratio of the CTAB method for these samples, with the exception of the maize snack, was above 1.5, which indicates the suitability of the extracted DNA for amplification analysis (Matsuoka et al. 2001; Querci et al. 2005). The purity of the extracts obtained from maize snack were quite low (A260/A280 ratios of ≤ 1.4), regardless of the extraction method, probably indicating some of presence of protein (Meyer 1999; Jasper et al. 2009). In the case of corn chips, the Wizard method enabled yields as high as the CTAB method. Conversely, the Wizard method gave not only statistically significantly higher DNA yields but also the best DNA purity (A260/A280 ratios of ≥ 1.7) in maize flour, maize starch, maize flakes and CRMs samples as verified by other others (Rizzi et al. 2003; Peano et al. 2004; Smith and Maxwell 2007). The mean of DNA yield and purity for the Genespin method in maize flour and CRMs was statistically similar to the Wizard method. This finding was also observed in the extraction of soybean flours and protein isolates (Mafra et al. 2008). Furthermore, Hrnčirova et al. (2008) demonstrated that the GeneSpin method was more effective for the extraction of DNA from raw materials. However, Mafra et al. (2008) verified that the Genespin and Nucleospin methods both produced high DNA yield and purity of soybean flour and simple products.

In the majority of samples tested both the Foodproof and DNeasy methods gave lower DNA yields than the other methods, although they still gave DNA of sufficient quality for PCR amplification (Table 2). In contrast, other authors described satisfactory results from DNA extracted by the DNeasy method for most food products (Di Pinto et al. 2007; Datukishvili et al. 2010).

Table 1 Statistical analysis of the DNA yield, DNA purities (A260/A280) and plant Ct values obtained by the five DNA extraction methods

Variable	Factors	DF ^a	F value ^b	P
DNA yield	Methods	4	1756.292	0.000
	Matrix	10	1038.558	0.000
	Interaction	44	436.379	0.000
DNA Purity	Methods	4	11.234	0.000
	Matrix	10	17.954	0.000
	Interaction	44	3.261	0.000
Ct value	Methods	4	285.931	0.000
	Matrix	10	948.711	0.000
	Interaction	44	56.668	0.000

^a Degrees of freedom

^b ANOVA F-statistic (high value indicates high variance between groups)

Table 2 Comparison of DNA yield, purity and real-time PCR results for a positive control plant gene in maize-containing foodstuffs and feeds extracted with different methods

	Extraction method	DNA yield (ng/ μ L)	DNA purity (A260/A280)	Real-time (+/total)	Ct
Flour	Foodproof	50.17 $\pm$ 5.8c	1.92 $\pm$ 0.1c	6/6	18.77 $\pm$ 0.88b
	GeneSpin	163.46 $\pm$ 8.9b	1.99 $\pm$ 0.1b	6/6	18.73 $\pm$ 0.51b
	Wizard	304.4 $\pm$ 21.8a	1.85 $\pm$ 0.1d	6/6	19.11 $\pm$ 0.71b
	DNeasy	15.13 $\pm$ 2.3d	2.10 $\pm$ 0.1a	6/6	20.75 $\pm$ 0.77a
	CTAB	170.0 $\pm$ 7.9b	1.73 $\pm$ 0.1e	6/6	21.11 $\pm$ 0.95a
Starch	Foodproof	8.6 $\pm$ 1.0b	1.16 $\pm$ 0.2d	6/6	21.20 $\pm$ 0.82d
	GeneSpin	10.3 $\pm$ 2.1b	1.59 $\pm$ 0.1ab	6/6	26.53 $\pm$ 0.55a
	Wizard	76.0 $\pm$ 3.6a	1.60 $\pm$ 0.1a	6/6	25.55 $\pm$ 0.44c
	DNeasy	1.9 $\pm$ 0.4d	1.58 $\pm$ 0.1ab	6/6	24.82 $\pm$ 1.10b
	CTAB	38.0 $\pm$ 0.6c	1.51 $\pm$ 0.1b	6/6	23.89 $\pm$ 0.88c
Bread	Foodproof	40.87 $\pm$ 5.11c	1.42 $\pm$ 0.2c	6/6	21.58 $\pm$ 0.83c
	GeneSpin	36.33 $\pm$ 4.5d	2.04 $\pm$ 0.1a	6/6	21.31 $\pm$ 0.48c
	Wizard	85.50 $\pm$ 5.7b	1.48 $\pm$ 0.1c	6/6	23.71 $\pm$ 0.73a
	DNeasy	7.29 $\pm$ 0.8e	1.48 $\pm$ 0.1c	6/6	21.35 $\pm$ 0.77c
	CTAB	129.44 $\pm$ 2.8a	1.57 $\pm$ 0.1b	6/6	23.16 $\pm$ 0.26b
Flakes	Foodproof	67.23 $\pm$ 2.1b	1.83 $\pm$ 0.1b	6/6	20.15 $\pm$ 0.75e
	GeneSpin	27.25 $\pm$ 2.4d	1.87 $\pm$ 0.1b	6/6	21.78 $\pm$ 0.32d
	Wizard	70.0 $\pm$ 0.63a	1.68 $\pm$ 0.1c	6/6	22.44 $\pm$ 0.65c
	DNeasy	4.68 $\pm$ 0.2 e	1.99 0.1a	6/6	23.51 $\pm$ 0.54b
	CTAB	55.92 $\pm$ 3.8c	1.67 $\pm$ 0.1c	6/6	24.65 $\pm$ 0.65a
Chips	Foodproof	55.82 $\pm$ 3.3b	1.73 $\pm$ 0.1b	6/6	20.47 $\pm$ 0.43e
	GeneSpin	50.81 $\pm$ 3.6c	2.03 $\pm$ 0.1a	6/6	20.10 $\pm$ 0.61d
	Wizard	71.93 $\pm$ 5.9a	1.75 $\pm$ 0.1b	6/6	22.80 $\pm$ 0.51b
	DNeasy	17.93 $\pm$ 2.6c	1.70 $\pm$ 0.1b	6/6	23.57 $\pm$ 0.61 a
	CTAB	69.74 $\pm$ 2.3a	1.68 $\pm$ 0.1b	6/6	21.16 $\pm$ 0.43 c
Maize-based biscuit	Foodproof	30.0 $\pm$ 2.6d	1.28 $\pm$ 0.1b	6/6	23.64 $\pm$ 0.97d
	GeneSpin	60.84 $\pm$ 7.3b	1.37 $\pm$ 0.1a	6/6	24.67 $\pm$ 0.63c
	Wizard	44.1 $\pm$ 6.5 c	1.38 $\pm$ 0.1a	6/6	25.95 $\pm$ 0.59b
	DNeasy	25.90 $\pm$ 3.9e	1.25 $\pm$ 0.1b	6/6	25.92 $\pm$ 0.51b
	CTAB	111.6 $\pm$ 4.7a	1.26 $\pm$ 0.1b	6/6	29.75 $\pm$ 0.37a
Diet breakfast cereal	Foodproof	71.35 $\pm$ 8.8d	1.52 $\pm$ 0.1b	6/6	21.46 $\pm$ 0.97c
	GeneSpin	117.63 $\pm$ 9.8c	1.76 $\pm$ 0.1a	6/6	20.34 $\pm$ 0.54d
	Wizard	273.7 $\pm$ 18.0b	1.52 $\pm$ 0.1b	6/6	23.90 $\pm$ 0.62a
	DNeasy	14.63 $\pm$ 4.4e	1.56 $\pm$ 0.1b	6/6	22.15 $\pm$ 0.83b
	CTAB	999.9 $\pm$ 93.7a	1.75 $\pm$ 0.1a	6/6	20.71 $\pm$ 0.71d
Canned-maize	Foodproof	89.85 $\pm$ 7.7c	1.58 $\pm$ 0.1c	6/6	21.46 $\pm$ 0.63 cd
	GeneSpin	29.43 $\pm$ 3.9d	1.71 $\pm$ 0.1b	6/6	21.75 $\pm$ 0.53c
	Wizard	103.7 $\pm$ 10.5b	2.10 $\pm$ 0.1a	6/6	24.49 $\pm$ 0.67a
	DNeasy	19.50 $\pm$ 2.5 e	2.15 $\pm$ 0.1a	6/6	23.24 $\pm$ 0.77b
	CTAB	203.37 $\pm$ 10.1a	1.63 $\pm$ 0.1c	6/6	20.97 $\pm$ 0.80d
Feed	Foodproof	36.98 $\pm$ 6.3d	1.93 $\pm$ 0.1a	6/6	22.19 $\pm$ 0.56c
	GeneSpin	63.99 $\pm$ 6.1c	1.96 $\pm$ 0.1a	6/6	21.02 $\pm$ 0.55d
	Wizard	103.7 $\pm$ 10.5b	1.67 $\pm$ 0.1c	6/6	22.87 $\pm$ 0.61b
	DNeasy	48.2 $\pm$ 3.6d	1.79 $\pm$ 0.1b	6/6	25.23 $\pm$ 1.18a
	CTAB	890.2 $\pm$ 40.9a	1.53 $\pm$ 0.1d	6/6	20.53 $\pm$ 0.10d
Seed	Foodproof	283.71 $\pm$ 38.8c	1.91 $\pm$ 0.1a	6/6	20.55 $\pm$ 0.65c
	GeneSpin	403.95 $\pm$ 60.2b	1.93 $\pm$ 0.1a	6/6	18.21 $\pm$ 0.92 b
	Wizard	73.3 $\pm$ 17.2d	1.81 $\pm$ 0.1b	6/6	21.10 $\pm$ 0.62 a

Table 2 (continued)

	Extraction method	DNA yield (ng/ μ L)	DNA purity (A260/A280)	Real-time (+/total)	Ct
CRMs	DNeasy	65.47 $\pm$ 13.8d	1.82 $\pm$ 0.1b	6/6	21.75 $\pm$ 0.36 b
	CTAB	532.4 $\pm$ 63.2	1.75 $\pm$ 0.1c	6/6	19.75 $\pm$ 0.54 b
	Foodproof	18.37 $\pm$ 81.6b	1.80 $\pm$ 0.1a	6/6	16.10 $\pm$ 0.41b
	GeneSpin	373.54 $\pm$ 47.7a	1.77 $\pm$ 0.1a	6/6	15.48 $\pm$ 0.33c
	Wizard	49.77 $\pm$ 26.7ab	1.78 $\pm$ 0.1a	6/6	15.65 $\pm$ 0.46d
	DNeasy	263.0 $\pm$ 46.1c	1.66 $\pm$ 0.1b	6/6	18.17 $\pm$ 0.57b
	CTAB	46.37 $\pm$ 51.0c	1.64 $\pm$ 0.1b	6/6	18.10 $\pm$ 0.39d

*Values represented as average $\pm$ standard deviation for six replicates

Effect of the DNA extraction methods on real time PCR efficiency All five DNA isolation methods performed well in providing DNA suitable for RT-PCR from the maize foodstuffs and feeds analyzed, as evidenced by the amplification of the plant-specific control gene. In general, higher Ct values (indicating smaller amounts of amplifiable plant derived-DNA) were obtained for seeds, food matrices and feeds than for CRM materials, for all extraction methods (Table 2). However, the significant differences in the Ct values observed indicates the importance of selecting appropriate extraction methods for different maize food matrices, as previously demonstrated by other authors

(Mafra et al. 2008; Fernandes et al. 2014). Furthermore, some differences in the efficiency of screening for GMO elements (P-35S, T-NOS, bar, FMV P-35S) were observed between the extraction methods tested. When the Wizard, DNeasy, Genespin and CTAB methods were applied to maize starch, the Foodproof kit to Diet Breakfast Cereal, and the Wizard and Dneasy kits to maize feed, no amplification was observed for GMO screening elements (35S/NOS/bar/FMV) in at least one of six replicates (Table 5) demonstrating that the same level of robustness can be obtained for GMO screening only if the appropriate DNA extraction procedure is used (Cankar et al. 2006).

Table 3 List of samples that tested positive for one or more GM elements

Sample	Extraction method	35S	NOS	Bar	FMV
Maize feed	Foodproof	32.49 $\pm$ 0.15	32.74 $\pm$ 0.20	ND	ND
	GeneSpin	32.44 $\pm$ 0.25	34.52 $\pm$ 0.44	ND	ND
	Wizard	31.98 $\pm$ 0.25*	30.72 $\pm$ 0.11	ND	ND
	DNeasy	30.75 $\pm$ 0.34*	32.30 $\pm$ 0.26*	ND	ND
	CTAB	31.12 $\pm$ 0.32	31.30 $\pm$ 0.71	ND	ND
Maize starch1	Foodproof	32.98 $\pm$ 0.55	33.05 $\pm$ 0.23	ND	ND
	GeneSpin	38.00 $\pm$ 0.36*	35.94 $\pm$ 0.42*	ND	ND
	Wizard	32.88 $\pm$ 0.31*	30.97 $\pm$ 0.13	ND	ND
	DNeasy	30.58 $\pm$ 0.47*	29.43 $\pm$ 0.17	ND	ND
	CTAB	30.95 $\pm$ 0.43*	28.91 $\pm$ 0.34	ND	ND
Maize starch2	Foodproof	ND	27.44 $\pm$ 0.31*	ND	ND
	GeneSpin	ND	30.32 $\pm$ 0.30*	ND	ND
	Wizard	ND	24.40 $\pm$ 0.15	ND	ND
	DNeasy	ND	28.63 $\pm$ 0.86	ND	ND
	CTAB	ND	30.18 $\pm$ 0.29	ND	ND
Diet breakfast cereal	Foodproof	ND	28.36 $\pm$ 0.32*	ND	ND
	GeneSpin	ND	28.99 $\pm$ 0.22	ND	ND
	Wizard	ND	27.87 $\pm$ 0.14	ND	ND
	DNeasy	ND	27.30 $\pm$ 0.15	ND	ND
	CTAB	ND	29.46 $\pm$ 0.23	ND	ND

For positive amplification signals, the mean Ct values and standard deviation are indicated

* no positive signals obtained at least for one of six replicates

ND no amplification detected

Table 4 Coefficient of correlation and efficiency of real-time PCR Standard curves obtained from serial diluted DNA extracts from maize flour (CRMs)

Target	Mean and SD*			
	Extraction method	Coefficient of correlation (R ²)	Angular coefficient (slope)	Efficiency**
Plant DNA	Foodproof	0.985±0.002	−3.197±0.71	105.6±3.3
	Genespin	0.991±0.006	−3.303±0.82	100.9±3.5
	Wizard	0.994±0.003	−3.446±0.10	96.6±2.0
	Dneasy	0.995±0.006	−3.381±0.45	97.6±1.6
	CTAB	0.996±0.005	−3.303±0.61	100.8±2.7

*SD standard deviation

**E, Efficiency, $E = ([10^{(-1/\text{slope})}] - 1) \times 100\%$

With regard to the dilution test (5 % Bt11 DNA), the correlation coefficient (R^2), slope of the regression line and the PCR efficiencies of DNA amplification from each of the five extraction methods were not statistically different from each other ($p > 0.05$) and in all cases distortion from the normal distribution was not observed with the Shapiro-Wilk Test for normality and Levene Test for uniformity of variances (Table 3). The higher degree of correlation of the calibration curves than the minimum acceptable coefficient ($R^2 > 0.98$) emphasises the adequacy of all extraction methods for quantification (Branquinho et al. 2010). Furthermore, the slightly lower R^2 values obtained from the Foodproof and Genespin methods suggests that, when DNA yields are lower, the presence of any impurities leads to greater variability in plant-derived DNA target amplification in these methods. The average PCR efficiencies of Wizard, Genespin and CTAB

methods were similar and close to 100 %. However, the amplification efficiency of Foodproof and DNeasy was higher than 100 %, a consequence of over-amplification, which could be explained by a compound or specific DNA structural conformation (Cankar et al. 2006). These results demonstrated that either the Wizard, Genespin or CTAB method were most efficient in providing amplifiable DNA from maize food and feed samples, indicating the suitability of these three extraction methods for real-time PCR quantification (Mafra et al. 2008; Mazzara et al. 2013).

Determination of the GMO present in maize food and feed products Seven CRMs (Bt11, Bt176, GA21, MON810, TC1507, NK603, MON89034) were tested to assess the specificity of the real-time multiplex PCR kit. The GMO screening by qualitative real-time PCR gave the expected results in each

Table 5 Results of screening for GM elements by qualitative RT-PCR in DNA extracted from commercial maize - containing foods and feeds and certified reference materials

Products	Number of samples	35S	NOS	BAR	FMV	PLANT
CRM						
(Bt11)	1	1	1	0	0	1
(Bt176)	1	1	0	1	0	1
(GA21)	1	0	1	0	0	1
(MON810)	1	1	0	0	0	1
(TC1507)	1	1	0	0	0	1
(NK603)	1	1	1	0	0	1
Food						
Flour	5	0	0	0	0	5
Starch	3	1	2	0	0	3
Bread	4	0	0	0	0	4
Breakfast cereal	4	0	0	0	0	4
Chips	4	0	0	0	0	4
Snack	4	0	0	0	0	4
Diet breakfast cereal	5	0	1	0	0	5
Canned	4	0	0	0	0	4
Feed	6	1	1	0	0	6
Seed	10	0	0	0	0	10
Total	55	7	7	0	0	55

CRM for the 35S/NOS/bar/FMV screening elements and the plant gene (Table 4), showing that the utility of a qualitative multiplex real-time PCR assay (the Foodproof GMO Screening Kit) for reliable screening of maize food and feed products (Dorries et al. 2010).

Of all 49 maize products screened, 45 (92 %) were negative for 35S/NOS/FMV GM elements. Nine samples (8 %) tested positive for the presence of one GM element (NOS), of which 2 samples (4 %) contained a second GM element (35S/NOS). GM material was found in two samples of maize starch and one each of diet breakfast cereal and maize feed, but not in any of the other foodstuffs (Table 4). The GMO screening results demonstrate the presence of GM material in maize foodstuffs and feeds that are commercially sold in local Turkish markets without any indication for consumers, in spite of the labelling regulations. The use of GM materials in food and feed industries was similarly reported by other others (Branquinho et al. 2010; Herzallah 2012). Conversely, our results differed from the recent report of Arun et al. (2013) in which 32.6 % of maize samples tested were positive for GMOs, as well as Gurakan et al. (2011) who found that 11 of the total 31 Turkish maize foodstuff samples (35 %) tested were GMO positive; but were similar to the results of Greiner and Konietzny (2008) who found 8–11 % of GM in 100 Brazilian maize products, which may indicate that the prevalence of GM maize material in food and feed products depends on the year. Similarly, the results of Herzallah (2012) showed that in Jordan, 5.4 % of the total food and feed tested samples were GM positive. The prevalence of GM materials in food and feed industries is the subject of ongoing studies in many countries (Tung-Nguyen et al. 2009; Wang et al. 2012; Herzallah 2012; Fernandes et al. 2014).

In our GMO-positive maize foodstuff samples, the consistency of amplification across all extraction methods indicates that GMO screening elements are present, albeit in small amounts, as screening for GM elements gave no amplification from GMO-negative samples or negative controls included on all RT-PCR plates. The relatively high Ct values of 35S/NOS/FMV gene targets in food samples suggests the presence only of trace amounts of GMO material in these products, which could be explained by unintentional contamination at the commodity shipment stage (Kluga et al. 2012). This suggests that they may be in compliance with labeling legislation since none of the analyzed foods declared the presence of GMO (Fernandes et al. 2014). However, the quantity of GM materials found in maize food and feed should be checked quantitatively to comply with the EU and international regulations stating that labelling is required when the content of GM material exceeds 0.9 % of food and feed products (Regulation (EC) No. 1829/2003).

Usefully, concerning the Foodproof GMO Screening Kit, the combination of screening targets present and absent in

positive samples gives information not only about the presence of a GMO, but also about the possible identity of the GMO present (Dorries et al. 2010). For example, in Turkey a maize sample with a positive signal for the NOS terminator should be further tested for the presence of the event GA21, and the event Bt11, NK603 or MON863 if the sample tested positive for both the CaMV 35S promoter and the NOS terminator.

Conclusions

DNA extraction methods should be evaluated for each food and feed matrix with regard to high DNA yield and purity as well as PCR efficiency. Of the methods, Wizard, the CTAB method and Genespin were found to be the most reliable DNA extraction methods for maize products since the best yields for all of the samples tested were achieved by one or more of these three methods, all of which produced high DNA purity and amplifiable DNA. To comply with labelling regulations in the EU and Turkey, GM materials in the food and feed chains must be traced; the qualitative multiplex real-time PCR assay used here is a simple and rapid detection tool for this purpose. Further investigation on quantification of GMOs would extend our knowledge and facilitate compliance with the 0.9 % threshold level for approved GMOs in the EU and Turkey.

Acknowledgments This research has been financially supported by Republic of Turkey Small and Medium Enterprises Development Organization (KOSGEB) and Elips Health Products Ltd.

References

- Arun OO, Yilmaz F, Muratoglu K (2013) PCR detection of genetically modified maize and soy in mildly and highly processed foods. *Food Control* 32:525
- Branquinho MR, Renata TB, Ferreira Paola CL (2010) Survey of compliance with labeling legislation in food containing GMOs in Brazil. *J Food Compos Anal* 23:220–225
- Bustin R and Nolan T (2004) Analysis of mRNA expression by Real-time PCR. In: *Real-time PCR: an essential guide*. Norfolk : Horizon Bioscience p. 125–184
- Cankar K, Stebih D, Dreo T, Zel J, Gruden K (2006) Critical points of DNA quantification by real-time PCR -effects of DNA extraction method and sample matrix on quantification of genetically modified organisms. *BMC Biotechnol* 6:37–51
- Datukishvili N, Gabriadze I, Kutateladze T, Karseladze M, Vishnepolsky B (2010) Comparative evaluation of DNA extraction methods for food crops. *Food Sci Technol* 45:1316–1320
- Di Bernardo G, Gaudio SD, Galderisi U, Cascino A, Cipollaro M (2007) Comparative evaluation of different DNA extraction procedures from food samples. *Biotechnol Prog* 23:297–301
- Di Pinto A, Forte V, Guastadisegni MC, Martino C, Schena FP, Tantillo GA (2007) Comparison of DNA extraction methods for food analysis. *Food Control* 18:76–80

- Dorries HH, Remus I, Gonewald A, Gronewald C, Jager KB (2010) Development of a qualitative, multiplex real-time PCR kit for screening of genetically modified organisms (GMOs). *Anal Bioanal Chem* 396(6):2055–2064
- European Commission Regulation (EC) (2003a) No 1829/2003 of the European parliament and of the council of 22 September 2003 on genetically modified food and feed. *Off J Eur Union* L268:1–23
- European Commission Regulation (EC) (2003b) No 1830/2003 of the European parliament and of the council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending directive 2001/18/EC. *Off J Eur Union* L268:24–28
- Fandke M (2002) Real-Time PCR Screening of Genetically Modified Organisms with the LightCycler Instrument http://www.roche-applied-science.com/wcsstore/RAS-Catalog-Asset-Store/Articles/BIOCHEMICA_2_02-p12-14.pdf. Accessed 10 February 2014
- Fernandes TJR, Oliveira MBP, Mafra I (2014) A survey on genetically modified maize in foods commercialised in Portugal. *Food Control* 35:338–344
- Gain report (2013) Agricultural Biotechnology Annual Report Turkey <http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20AnnualAnkara%20Turkey%207-16-2013.pdf>. Accessed 10 February 2014
- Greiner R, Konietzny U (2008) Presence of genetically modified maize and soy in food products sold commercially in Brazil from 2000 to 2005. *Food Control* 19:499–505
- Gryson N (2010) Effect of food processing on plant DNA degradation and PCR-based GMO analysis: a review. *Anal Bioanal Chem* 396:2003–2022
- Gryson N, Messens K, Dewettinck K (2004) Evaluation and optimisation of five different extraction methods for soy DNA in chocolate and biscuits. extraction of DNA as a first step in GMO analysis. *J Sci Food Agric* 81:231–234
- Gurakan GC, Aydın G, Yılmaz R (2011) Qualitative detection GM maize (Bt11) in food and feed commercially sold in Turkey by PCR based methods. *Indian J Biotechnol* 10:14–146
- Herzallah SM (2012) Detection of genetically modified material in feed and foodstuffs containing soy and maize in Jordan. *J Food Compos Anal* 26:169–172
- Hrnčirova Z, Bergerova E, Siekel P (2008) Effects of technological treatment on DNA degradation in selected food matrices of plant origin. *J Food Nutr Res* 47(1):23–28
- ISO (2005) International standard 21571, foodstuffs—methods of analysis for the detection of genetically modified organisms and derived products—nucleic acid extraction. (Annex A.3) 2005. International Organization for Standardization, Geneva
- James C (2012) Global status of commercialized biotech/GM crops: *ISAAA brief* 44 New York. ISAAA, Ithaca
- Jasper K, Son R, Mohammad Ghazali F, Cheah YK (2009) Real-time PCR evaluation of seven DNA extraction methods for the purpose of GMO analysis. *Int Food Res J* 16:329–341
- Kluga L, Folloni S, Van den Bulcke M, Van den Eede G, Querci M (2012) Applicability of the “real-time PCR-based ready-to-use multi-target analytical system for GMO detection” in processed maize matrices. *Eur Food Res Technol* 234:109–118
- Mafra I, Silva SA, Moreira EJMO, Ferreira da Silva CS, Beatriz M, Oliveira PP (2008) Comparative study of DNA extraction methods for soybean derived food products. *Food Control* 19:1183–1190
- Matsuoka T, Kuribara H, Akiyama H, Miura H, Goda Y, Kusakabe Y, Isshiki K, Toyoda M, Hino A (2001) A multiplex PCR method of detecting recombinant DNAs from five lines of genetically modified maize. *J Food Hygienic Soc Japan* 42:24–32
- Mazzara M, Paoletti P, Corbisier P, Grazioli E, Larcher S, Berben G, De Loose M, Folch I, Henry C, Hess N, Hougs L, Janssen E, Moran G, Onori R, Van den Eede G (2013) Kernel lot distribution assessment (KeLDA): a comparative study of protein and DNA-based detection methods for GMO testing. *Food Anal Methods* 6:210–220
- Meyer R (1999) Development and application of DNA analytical methods for the detection of GMOs in food. *Food Control* 10(6):391–399
- Miraglia M, Berdal KG, Brera C, Corbisier P, Holst-Jensen A, Kok EJ (2004) Detection and traceability of genetically modified organisms in the food production chain. *Food Chem Toxicol* 42:1157–1180
- Peano C, Ruijter JM, Deprez RHL, Moorman AFM (2004) Qualitative and quantitative evaluation of the genomic DNA extract from GMO and non-GMO foodstuffs with four extraction methods. *J Agric Food Chem* 52:6962–6968
- Pirondini A, Bonas U, Maestri E, Visioli G, Marmioli M, Marmioli N (2010) Yield and amplifiability of different DNA extraction procedures for traceability in the dairy food chain. *Food Control* 21:663–668
- Querci M, Jermini M, Van den Eede G (eds) (2005) Users manual – training course on the analysis of food and feed samples for the presence of genetically modified organisms. Joint Research Centre, Ispra
- Querci M, Van den Bulcke M, Zel J, Van den Eede G, Broll H (2009) New approaches in GMO detection. *Anal Bioanal Chem* 396:1991–2002
- Rizzi A, Panebianco L, Giaccu D, Sorlini C, Daffonchio D (2003) Stability and recovery of maize DNA during food processing. *Ital J Food Sci* 15:499–510
- Smith DS, Maxwell PW (2007) Use of quantitative PCR to evaluate several methods for extracting DNA from corn flour and cornstarch. *Food Control* 18:236–242
- Tung-Nguyen CT, Son R, Raha AR, Lai OM, Clemente Michael WVL (2009) Comparison of DNA extraction efficiencies using various methods for the detection of genetically modified organisms (GMOs). *Int Food Research J* 16:21–30
- Wang X, Teng D, Yang Y, Tian F, Guan Q, Wang J (2012) Comparison of three DNA extraction methods for feed products and four amplification methods for the 5′-junction fragment of roundup ready soybean. *J Agric Food Chem* 60:4586–4595
- Zel J, Mazzara M, Savini C, Cordeil S, Camloh M, Stebih D, Cankar K, Grudem K, Morisset D, Van den Eede G (2008) Method validation and quality management in the flexible scope of accreditation: an example of laboratories testing for genetically modified organisms. *Food Anal Methods* 1:61–72