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Qualitative and Quantitative Evaluation of the Genomic DNA Extracted from GMO and Non-GMO Foodstuffs with Four Different Extraction Methods

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The presence of DNA in foodstuffs derived from or containing genetically modified organisms (GMO) is the basic requirement for labeling of GMO foods in Council Directive 2001/18/CE (*Off. J. Eur. Communities* **2001**, *L1 06/2*). In this work, four different methods for DNA extraction were evaluated and compared. To rank the different methods, the quality and quantity of DNA extracted from standards, containing known percentages of GMO material and from different food products, were considered. The food products analyzed derived from both soybean and maize and were chosen on the basis of the mechanical, technological, and chemical treatment they had been subjected to during processing. Degree of DNA degradation at various stages of food production was evaluated through the amplification of different DNA fragments belonging to the endogenous genes of both maize and soybean. Genomic DNA was extracted from Roundup Ready soybean and maize MON810 standard flours, according to four different methods, and quantified by real-time Polymerase Chain Reaction (PCR), with the aim of determining the influence of the extraction methods on the DNA quantification through real-time PCR.

KEYWORDS: Food traceability; GMO; DNA extraction; DNA degradation; real-time PCR

INTRODUCTION

The use of high-quality raw material in food production is considered to be a prerequisite factor to obtain a genuine and secure product of adequate nutritional value. In the European Community traceability of the origin, quality, and authenticity of food products is becoming very important (*1*). Consequently, it will become necessary to develop appropriate techniques to trace and label foods correctly (*2*). In this context, to avoid alimentary frauds, there is a need to develop reliable detection methods (*3*).

In the European Union (EU), labeling of foods derived from genetically modified crops has become one of the main issues of food safety dominating public discussion. The general public has shown anxiety over these novel foods, and pressure from consumer groups and public demand have led several countries to require labeling of genetically modified organisms (GMO) in foods (*4*). At the same time the food industry needs information on the presence and content of GM crops in raw materials. Therefore, the development of practical methods for detecting recombinant DNA (r-DNA) or its product protein in foods and raw materials is required in many countries.

Up to now, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) have been of minor interest in food analysis, and there is therefore little knowledge about the detection of nucleic acid (NA) in many processed foodstuffs.

After the approval and cultivation of various genetically modified crops in the United States and Europe in recent years, nucleic acids have become an important tool in food analysis (*5*, *6*). The reason in most cases is the discrimination between genetically modified or unmodified foodstuff that can best be achieved directly at the DNA level (*7*).

To develop appropriate techniques capable of distinguishing between genetically modified and non-modified foodstuffs and to prove the presence or absence of the introduced gene(s) at the level of DNA is a new goal in food analysis (*8*). Consequently, DNA must be extracted from the samples prior to analysis.

Although many DNA extraction protocols are available, they have been rarely compared in a comprehensive manner (*9*).

In this study four commercial kits of DNA extraction were compared and evaluated from both qualitative and quantitative points of view. The four methods of extraction were evaluated for (i) the amount of genomic DNA extracted, (ii) the degradation of the DNA extracted, and (iii) the effect on DNA quantification by real-time Polymerase Chain Reaction (PCR).

MATERIALS AND METHODS

Plant and Food Material. For accurate measurements, the same set of samples was utilized throughout the work. This includes some

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Table 1. Characteristics of the Food Samples Used

food sample	description	name
cracker (soybean)	bread substitute	Panlight of rice and soybean
cracker (maize)	bread substitute	Panlight of rice and maize
polenta	flour	polenta Bramata
tacos	snack food	taco dinner kit
tofu	vegetarian meat substitute	tofu

Table 2. Sequences of the Primers Designed on the Lectin and Zein Genomic Sequence

primer	sequence
SL71	5′GCAGCAGCAGCAGCAGACTA3′
SL633	5′CAAACCACACATAAGAGAGGATGG3′
SL155	5′GCCGAAGCAACCAAACATG3′
SL256	5′CTCTACTCCACCCCCATC3′
SL1	5′ATGGGCTTGCCTTCTTTCTC3′
SL2	5′CCGATGTGTGGATTTGGTG3′
SL520	5′GATGGATCTGATAGAATTGAC3′
SL1697	5′GGCAGCAGAGAACCCTATCCTC3′
zein deg F	5′TACAAGGATGCGATACACACA3′
zein 133R	5′TATGATGGTATGTCATTGCGC3′
zein 1	5′GCTTGCATTGTTCGCTCTC3′
zein 3	5′AGTGCGACCCATATTCCAG3′
zein 4	5′GACATTGTGGCATCATTATTT3′
zein 2	5′CGATGGCATGTCAACTCATTA3′
zein 1105R	5′AAGAGCTAGGAGAGCGAACAA3′
zein 1578R	5′AATAATGAGTCACACGCGATG3′
zein 1956R	5′CGTGACTGCTTCTTTTACGAT3′

selected maize- and soybean-derived food products purchased at local supermarkets, representing different food processing levels (**Table 1**). Certified maize (IRMM 413) and soybean (IRMM 410-S) reference materials obtained commercially (Fluka), and certified by the Institute for Reference Materials and Measurements (IRMM), were used as positive controls.

Genomic DNA Extraction. Food samples were pooled to reduce the sample volumes to a laboratory-scale quantity. Genomic DNA was extracted from 200 mg of these pooled materials. The samples were processed using the following methods: Wizard (Promega) (*9*), DNeasy Plant Minikit (*9*−*11*), QIAamp DNA Stool Minikit (*12*), and Nucleo Spin Food (Macherey-Nagel) (*13*). In these procedures, the cellular components of the sample were first lysed. In later steps, the DNA is bound to a membrane gel matrix, washed thoroughly, and then eluted according to the manufacturer's instruction.

Genomic DNA Quantification. The extracted DNA was analyzed prior to PCR analysis by agarose gel electrophoresis (*14*) with λ DNA digested with *Hin*dIII as molecular weight marker. For the determination of the approximate amount and average size of isolated DNA, aliquots of 5 μL were loaded onto 0.8% agarose gel and electrophoresed in 1× TAE buffer (*14*). The purity of the DNA content in the solution was checked on the basis of UV absorption ratio at 260/280 nm. The absorbance ratios at 260/280 nm obtained from soybean and maize extracts (respectively) were as follows: for the Nucleo Spin Food (1.9 ÷ 2.0; 1.6 ÷ 2.0), for the QIAamp DNA Stool Minikit (1.9 ÷ 2.0; 1.8 ÷ 2.0), for the Wizard (1.4 ÷ 2.0; 1.8 ÷ 2.0), and for the DNeasy Plant Minikit (1.7 ÷ 2.0). The DNA samples were subsequently used for PCR analysis.

Primers Design for the DNA Degradation Analysis. The primers, synthesized by MWG Biotech, were designed using Software Primer 1.2 for Macintosh on the genomic sequences of the lectin gene (GenBank accession K00821 and M30884) and on the genomic sequence of the zein gene (GenBank accession M23537) (**Table 2**).

Qualitative and Quantitative PCR. Extracted DNA was analyzed for its amplification by routine PCR using species and GMO-specific detection protocols. Prescreening was performed by amplifying DNA with generic plant-specific primers (for a chloroplast gene) to evaluate the amplification of the purified DNA (data not shown). Control

Table 3. Primer Pairs Used in Lectin Gene Degradation Study

	primer	annealing T (°C)	amplicon length (bp)
A	SL1/SL2	62	169
B	SL256/SL520	58	263
C	SL155/SL520	58	391
D	SL633/SL520	60	857
E	SL71/SL1697	64	1626

Table 4. Primer Pairs Used in Zein Gene Degradation Study

	primer	annealing T (°C)	amplicon length (bp)
A	zein deg F/zein 133	62	133
B	zein 3/zein 4	62	277
C	zein 1/zein 2	62	485
D	zein deg F/zein 1105	62	1105
E	zein deg F/zein 1578	62	1578
F	zein deg F/zein 1956	62	1956

reactions were performed using the primer pairs SL1/SL2 and zein3/zein4 targeting the intrinsic genes of soybean lectin and maize zein, respectively (*15*, *16*). DNA degradation was evaluated with an assay based on the decreasing amplicon size obtained by using the primer pairs listed in **Tables 3** and **4**, designed according to the sequences in **Table 2**. All PCRs were carried out on a RoboCycler Gradient 96 Thermal Cycler (Stratagene) with hot lid assembly. For the purpose of standardization, master mixes were prepared as described (*14*). All PCR assays were performed in final volumes of 25 μL containing 1× reaction buffer (Amersham Pharmacia), 3 mmol/L $MgCl_2$, 0.625 mmol/L of each primer, 0.5 mmol/L of dNTPs, 0.5 unit of Taq polymerase (Amersham Pharmacia), and 100 ng of genomic DNA. The amplification reactions were run under the following conditions: initial denaturation at 95 °C for 5 min; 40 cycles of denaturation (50 s at 95 °C); primer annealing for 50 s, annealing temperatures are shown in **Tables 3** and **4**, and their extension (1 min at 72 °C), with a terminal extension of 72 °C for 3 min. The amplification products were separated using a 1.5% agarose gel (Q-biogene) in 1× TAE buffer (*14*) stained with 1 μg/mL of ethidium bromide solution and visualized by using a UV Bio-Rad Gel Doc 2000 image detector.

Real-time PCR was also performed in parallel with the detection and validation for an accurate quantitative measurements of transgenic content in the DNA samples (*17*). The PCR reactions were carried out on a GeneAmp 5700 Sequence Detector System (Applied Biosystems) using TaqMan chemistry. The real-time PCR mix contained 1× TaqMan Universal Mastermix (Applied Biosystems), 0.52 μM primers, 0.156 μM probe, and 250 ng of template DNA, making a final volume of 25 μL.

The system runs an initial incubation at 50 °C for 2 min to allow uracil DNA glycosylase (UDG) to digest any amplicon carry-over, followed by 95 °C for 10 min to inactivate the UDG, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and a final elongation stage of 7 min at 72 °C. All reactions were run in triplicate. The structures of primers and probes utilized are given in **Table 5**.

RESULTS AND DISCUSSION

Genomic DNA Extraction. DNA was extracted from foodstuffs chosen on the basis of the complexity of their composition and the technological treatment they underwent (*10*).

Foodstuffs containing maize ingredients used in this study were the following: seeds (no treatment), flours and polenta (mechanical treatment), crackers (mechanical and thermal treatment), and tacos (mechanical, thermal, and chemical treatment) (**Table 1**). Maize crackers and tacos, moreover, contain ingredients capable of inhibiting PCR such as fatty acids and oils.

Table 5. Sequences of Primers and Probes Used in Real-Time PCR

name	specificity	sequence 5′–3′	ref
CaMV3	35S promoter	5′-GTCTTGCGAAGGATAGTGGGA-3′	*18*
CaMV4	35S promoter	5′-CACGTCTTCAAAGCAAGTGGA-3′	*18*
LectF	lectin gene	5′-TCCACCCCCATCCACATTT-3′	*18*
LectR	lectin gene	5′-GGGATAGAAGGTGAAGTTGAAGGA-3′	*18*
ZeinF	zein gene	5′-GCATGATGCAACAAGGGCTT-3′	*18*
ZeinR	zein gene	5′-AGGCCAACAGTTGCTGCAG-3′	*18*
CaMV probe	35S promoter	5′FAM-TGCGTCATCCCTTACGTCAGTGGAGAT-TAMRA 3′	*18*
Lect probe	lectin gene	5′FAM-AACCGGTAGCGTTGCCAGCTTGT-TAMRA 3′	*18*
Zein probe	zein gene	5′FAM-TTGATGGCGTGTCCGTCCCTGA-TAMRA 3′	*18*

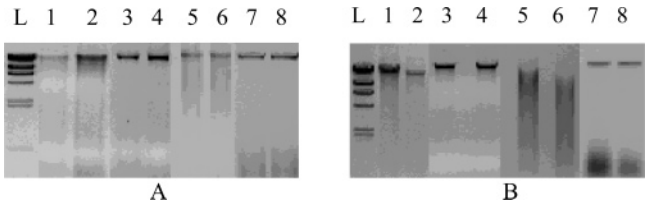

Figure 1. Agarose gel electrophoresis of genomic DNA extracted from maize seeds and standard flours (**A**) and soybean standard flours (**B**): lane L, molecular marker λ *Hin*dIII; lanes 1 and 2, WR extraction; lanes 3 and 4, DNeasy extraction; lanes 5 and 6, NSF extraction; lanes 7 and 8, DNA Stool extraction. Two extractions for each sample were performed.

Foodstuffs containing soybean ingredients used were the following: flours (mechanical treatment), crackers (mechanical and thermal treatment), and tofu (mechanical, thermal, and chemical treatment). Crackers and tofu contain ingredients capable of inhibiting PCR (**Table 1**).

DNA was extracted with the four extraction kits analyzed, and the quantity of DNA obtained was evaluated spectrophotometrically and by gel electrophoresis (*14*).

The quantification of DNA on agarose gel was achieved only when DNA was extracted from those foodstuffs that underwent only mechanical treatments such as seeds, flours, and polenta. In these cases it has been possible to detect the band corresponding to the genomic DNA following agarose gel electrophoresis (**Figure 1**).

Using the DNA obtained from other foodstuffs, it was impossible to perform genomic quantification on agarose gel because of the absence of the band corresponding to the genomic DNA, from which it can be inferred that the DNA extracted was substantially degraded.

Amounts of genomic DNA obtained from the four extraction methods were analyzed spectrophotometrically and the results compared (**Figure 2** and **3**).

From the two histograms it is evident that the QIAamp DNA Stool Minikit (*12*) and the Wizard (Promega) (*9*) extraction kit gave the highest yield of genomic DNA from simple foodstuffs such as seeds and flours, whereas the Nucleo Spin Food kit (Macherey-Nagel) (*13*) gave the highest yield of DNA from complex foodstuffs such as crackers, tacos, and tofu.

Degradation Study. The quality of the DNA extracted from food samples is generally influenced by these three factors: (a) the presence of PCR inhibitors in the food matrices; (b) the grade of damage (e.g., depurination) of the DNA; and (c) the average fragment length of the NA extracted. These factors are dependent on the sample itself, the processes carried out during the production of the food, and the physical and chemical parameters of the extraction method utilized. Exposure to heat is known to cause fragmentation of high molecular weight DNA (*18*, *19*), and physical or chemical treatments will cause random breaks in DNA strands, thus reducing the average DNA fragment size.

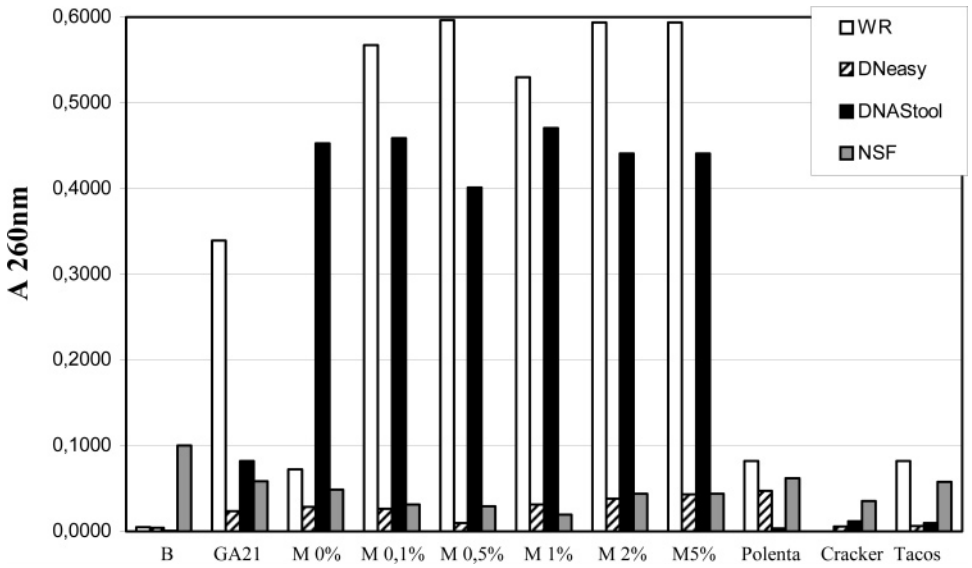

Figure 2. Spectrophotometric quantification of DNA extracted from maize foodstuffs. Genomic DNA was extracted from GA21 seeds, from MON810 standard flours, and from polenta, maize crackers, and tacos with the four extraction methods analyzed: NSF, DNA Stool, WR, and DNeasy. The quantity of DNA purified was evaluated by UV absorption at 260 nm. B, no DNA; M0–5%, standard flours of maize containing increasing percentages of MON810 transgene.

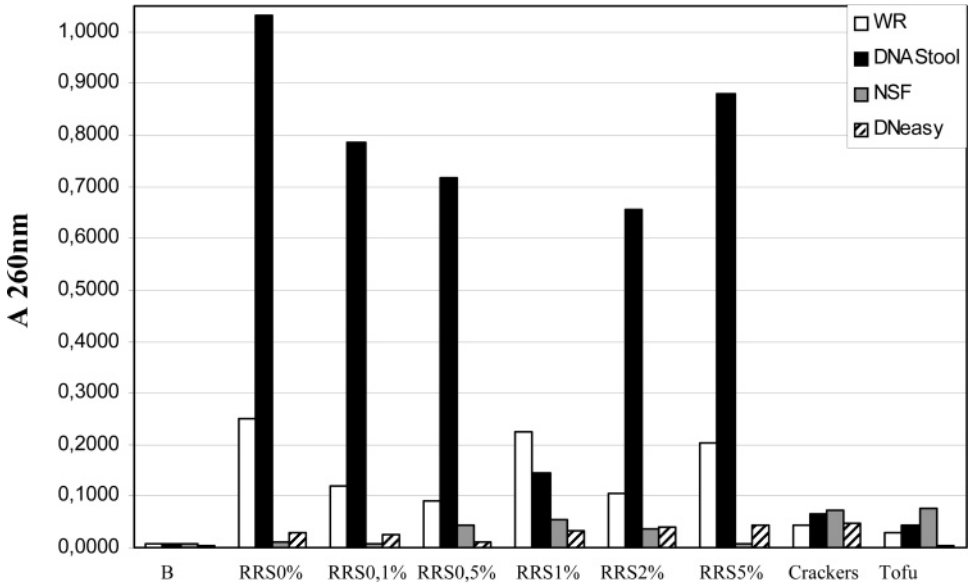

Figure 3. Spectrophotometric quantification of DNA extracted from soybean foodstuffs, with the methods analyzed. Genomic DNA was extracted form RRS standard flours and from soybean crackers and tofu with the four extraction methods: NSF, DNA Stool, WR, and DNeasy. The quantity of DNA purified was evaluated by UV absorption at 260 nm. B, no DNA; RRS0–5%, standard flours of soybean containing increasing percentages of RRS transgene.

Many foods, such as fruits and vegetables, are characterized by their acidity, thus accelerating the acid-catalyzed reactions in the course of thermal treatments. On the other hand, processing at alkaline pH is part of the production of other foods; a typical example is the use of strongly alkaline solutions in the initial stages of the preparation of tacos and other similar foods from maize. The DNA is very sensitive to acid and alkaline agents because of the mechanism of hydrolytic degradation of DNA. At acid pH, purines are removed from the nucleic acid backbone due to the cleavage of N-glycosidic bonds between deoxyribose residues and bases. Subsequently, adjacent 3′,5′-phosphodiester linkages are hydrolyzed, leading to the shortening of DNA strands (*20*, *21*).

The DNA samples extracted from foodstuffs containing soybean and maize material, with the four extraction kits, were evaluated as far as their degradation levels were concerned.

For this purpose five primer pairs (**Tables 2** and **3**) were designed on the genomic sequence of the lectin gene, and six primer pairs (**Tables 2** and **4**) were designed on the genomic sequence of the zein gene.

The primer pairs designed on the lectin gene were able to amplify the following fragments: A, 169 bp; B, 263 bp; C, 391 bp; D, 857 bp; E, 1626 bp. The primer pairs designed on the zein gene were able to amplify the following fragments: A, 133 bp; B, 277 bp; C, 485 bp; D, 1105 bp; E, 1578 bp; F, 1956 bp.

These primer pairs were used to amplify DNA extracted from all of the soybean and maize foodstuffs, and the results of this analysis are shown in **Figures 4** and **5**.

Considering the lengths of the amplicons obtained as the criterion for DNA integrity, the largest amplicons were obtained in these conditions: 1956 bp for maize seeds obtained from all of the extraction kits with the exception of the DNeasy Plant Minikit (**Figure 5**); 1578 bp for maize standard flour obtained from all of the extraction kits with the exception of DNeasy Plant Minikit (**Figure 5**); 1626 bp for soybean standard flour obtained from all of the extraction kits (**Figure 4**); 1956 bp for polenta with the NucleoSpin Food (Macherey-Nagel) kit, 1105 bp with QIAamp DNA Stool Minikit and Wizard kit (Promega),

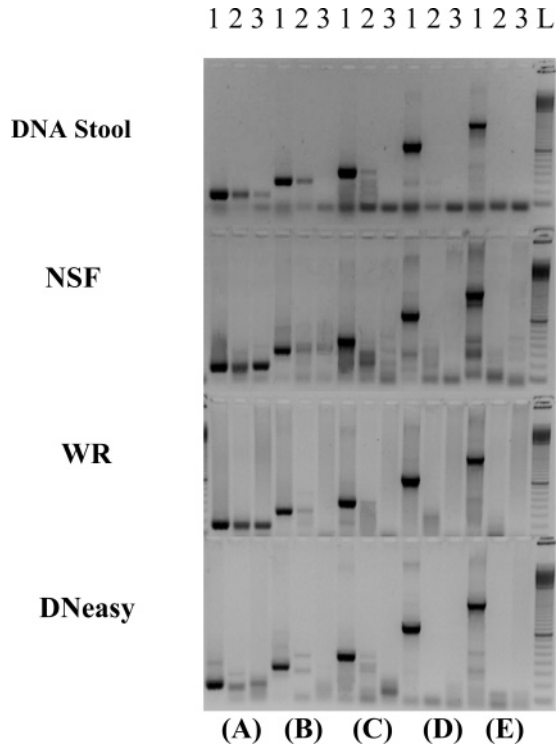

Figure 4. Agarose gel electrophoresis of PCR products obtained from genomic DNA of soybean-based foodstuffs extracted with the four methods: lane L, 100 bp molecular marker; lane 1, RRS standard flour 1%; lane 2, soybean crackers; lane 3, tofu. The primer pairs (A–E) used for the PCR are listed in **Table 3**.

and only 485 bp with the DNeasy Plant Minikit (**Figure 5**); 133 bp for maize crackers with the DNeasy Plant Minikit and Wizard (Promega), 277 bp with the QIAamp DNA Stool Minikit, and 485 bp with the Nucleo Spin Food (Macherey-Nagel) (**Figure 5**); 391 bp for soybean crackers obtained from all of the methods analyzed (**Figure 4**); 133 bp for tacos with the QIAamp DNA Stool Minikit and 485 bp with Nucleo Spin Food (Macherey-Nagel) (**Figure 5**); 169 bp for tofu obtained

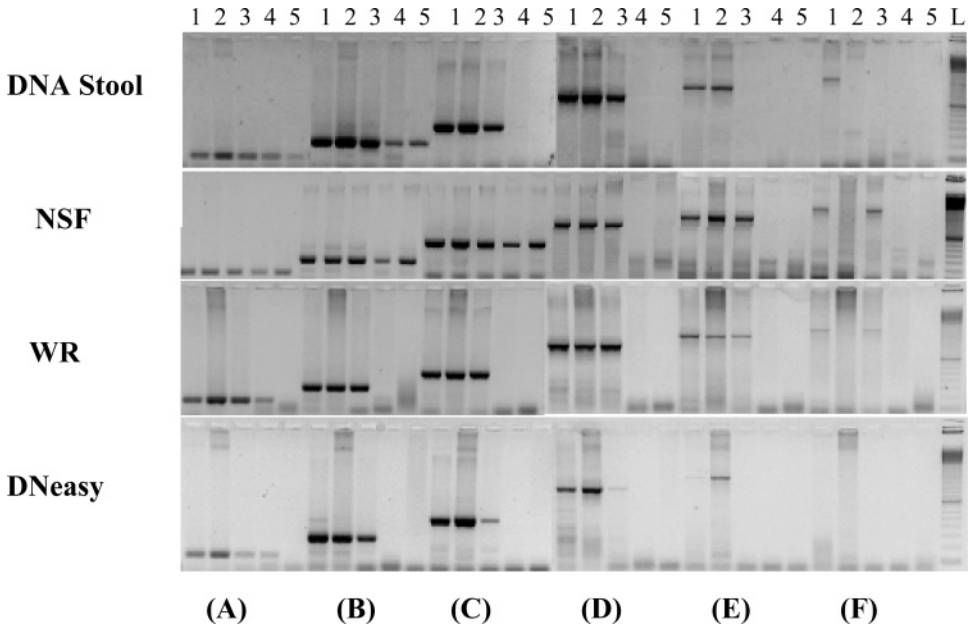

Figure 5. Agarose gel electrophoresis of PCR products obtained from genomic DNA of maize-based foodstuffs extracted with the four methods: lane L, 100 bp molecular marker; lane 1, maize seeds; lane 2, MON810 standard flour 1%; lane 3, polenta; lane 4, crackers; lane 5, tacos. The primer pairs (A–F) used for the PCR are listed in **Table 4**.

from all of the methods analyzed except with the Nucleo Spin Food (Macherey-Nagel) (**Figure 4**).

From these results it is evident that the treatments of the foodstuffs can affect the DNA degradation level, and at the same time it is also evident that the method of extraction can have a great influence on DNA degradation and/or yield and/or PCR amplification efficiency on degraded DNA.

Among the analyzed methods of extraction, the QIAamp DNA Stool Minikit gave a good-quality DNA with a very low level of degradation from simple foodstuffs; the Nucleo Spin Food kit (Macherey-Nagel) proved to be the most efficient in recovering good-quality DNA with a low level of degradation from complex foodstuffs.

Relevance of the Extraction Methods on Quantification of Transgenes by Real-Time PCR. An important aspect of the GMO evaluation in food analysis is quantification, because the maximum limit of GMO in foods and ingredients is fixed at 0.9% by EU Directive 2001/18. Any value higher than this limit leads to labeling. Real-time PCR is widely recognized as the most reliable quantitative tool for the determination of GMO content in foods. The ability to monitor the progress of the PCR in real time completely revolutionized the approach to PCR-based quantitation of DNA and RNA. Compared to other endpoint quantitation methods, real-time PCR offers a streamlined assay development, reproducible results, and a large dynamic range, and it has good specificity and sensitivity (*22*).

Following the findings illustrating that the extraction method used in genomic DNA purification from foodstuffs has a great influence both on the quantity and on the quality of the DNA obtained, the influence of the four extraction methods on the performance of real-time PCR was also evaluated. Due to the sensitivity of the technique, it is important to ensure that the DNA is pure and free from PCR inhibitors (*17*, *23*).

DNA from RRS (IRMM 410-S) and maize MON810 (IRMM 413) standard flours containing 0, 0.1, 0.5, 1, 2, and 5% of transgenic material and extracted with the four methods was quantified in real-time PCR. The probe used for the quantification was complementary to the CaMV 35S promoter (**Table 5**) present in both of the transgenic constructs considered (*18*, *24*).

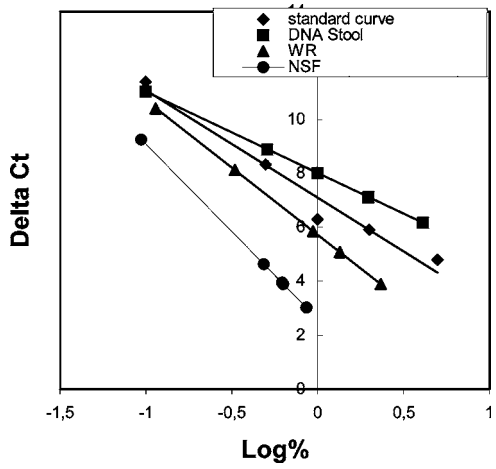

Figure 6. Fitting between mathematical and standard curves of quantification of DNA extracted from RRS standard flours with the four methods analyzed. The graph shows the comparison of the mathematical standard curve and the standard curves deriving from the quantification by RT-PCR of the DNA extracted from RRS standard flours with three of the extraction methods considered: DNA Stool, WR, and NSF.

These samples were contextually tested for their reference gene content (i.e., lectin in the case of soybean and zein in the case of maize). The results obtained from the quantification of GMO specific sequences and of plant endogenous genes were correlated to obtain a relative quantification of the transgenic content from two absolute quantifications during the analysis of results.

The quantities of transgenic constructs determined by real-time PCR (**Figures 6** and **7**) were compared with the standard quantities, and a statistical analysis based on Student's *t* test for coupled samples (**Tables 6** and **7**) was performed to determine which of the four extraction methods was more appropriate for use in real-time PCR analysis.

The quantification of the transgenic content of the standard flours in RRS (**Table 6**) extracted with the QIAamp DNA Stool Minikit appeared to be the one offering the highest correlation

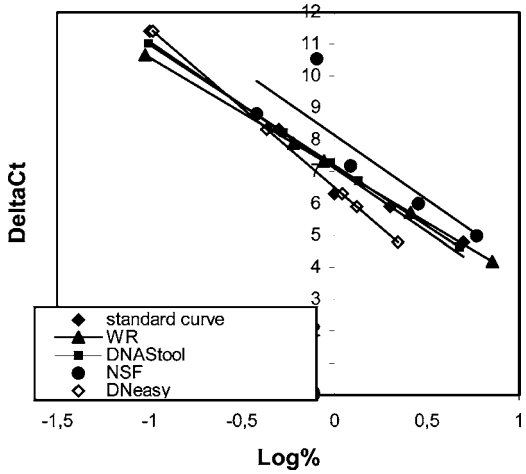

Figure 7. Fitting between mathematical and standard curves of quantification of DNA extracted from maize MON810 standard flours with the four methods analyzed. The graph shows the comparison of the mathematical standard curve and the standard curves deriving from the quantification by real-time PCR of the DNA extracted from MON810 standard flours with the methods considered: DNA Stool, WR, NSF, and DNeasy.

Table 6. *t* test for Coupled Samples and Statistical Analysis of the Best Fitting between Mathematical and Standard Curves of Quantification of DNA Extracted from RRS Standard Flours with the Different Methods Analyzed[a]

method	r^2	$P(r^2)$	t	$P(t)$
DNAStool	0.987	0.002 **	–2.416	0.073 ns
WR	0.900	0.038 *	1.285	0.268 ns
NSF	0.841	0.074 ns	3.736	0.020 *

[a] r^2 = correlation; $P(r^2)$ = significance of r^2; t = t coefficient; $P(t)$ = significance of t; *** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$; ns = $P > 0.05$.

Table 7. *t* Test for Coupled Samples and Statistical Analysis of the Best Fitting between Mathematical and Standard Curves of Quantification of DNA Extracted from Maize MON810 Standard Flours with the Different Methods Analyzed[a]

method	r^2	$P(r^2)$	t	$P(t)$
DNAStool	0.976	0.004 **	–0.520	0.630 ns
WR	0.962	0.009 **	0.611	0.574 ns
NSF	0.896	0.040 *	–3.399	0.027 *
DNeasy	0.975	0.005 **	–0.809	0.464 ns

[a] r^2 = correlation; $P(r^2)$ = significance of r^2; t = t coefficient; $P(t)$ = significance of t; *** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$; ns = $P > 0.05$.

with the expected values, whereas the quantification of the transgenic content of the standard flours in MON810 (**Table 7**) extracted with DNA Stool, WR, and DNeasy kits showed the same significance of correlation with the expected values for which they showed the highest correlation.

This analysis shows that the extraction methods, determining the amount, integrity, and purity of the DNA, can have a great influence also on the results obtained by real-time PCR. Therefore, it is extremely important to use, for each food matrix, the DNA extraction method that correlates best with performance of subsequent DNA analysis, such as real-time PCR testing.

ABBREVIATIONS USED

RRS, Roundup Ready soybean; MON810, maize Monsanto 810; PCR, Polymerase Chain Reaction; NA, nucleic acids; DNA, deoxyribonucleic acid; CaMV, cauliflower mosaic virus; IRMM, Institute for Reference Materials Measurements; GMO, genetically modified organism; RT-PCR, real-time PCR; EU, European Union; bp, base pair; P-35S, promoter 35S of the cauliflower mosaic virus; DNA Stool, QIAamp DNA Stool Minikit; NSF, Nucleo Spin Food (Macherey-Nagel); WR, Wizard (Promega); DNeasy, DNeasy Plant Minikit.

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