Real-Time Quantitative PCR Detection of Genetically Modified Maximizer Maize and Roundup Ready Soybean in Some Representative Foods

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A fast and quantitative method was developed to detect transgenic "Maximizer" maize "event 176" (Novartis) and "Roundup Ready" soybean (Monsanto) in food by real-time quantitative PCR. The use of the ABI Prism 7700 sequence detection system allowed the determination of the amplified product accumulation through a fluorogenic probe (TaqMan). Fluorescent dyes were chosen in such a way as to coamplify total and transgenic DNA in the same tube. Using real-time quantitative PCR, 2 pg of transgenic or total DNA per gram of starting sample was detected in 3 h after DNA extraction and the relative amounts of "Maximizer" maize and "Roundup Ready" soybean in some representative food products were quantified.

Keywords: *"Maximizer" maize "event 176"; "Roundup Ready" soybean; real-time quantitative PCR; GMO detection*

INTRODUCTION

The rapid development of biotechnology has launched products and ingredients derived from genetically modified organisms (GMOs) into the food market, which society is usually not very familiar with (Biotechnology and the European Public Concerted Action group, 1997). Information and transparency for the acceptance of these new products by consumers are essential. A key factor in this issue is the availability of methods to distinguish between transgenic food and their traditional counterparts. Several analytical methods using polymerase chain reaction (PCR) technology have been developed to qualitatively detect the presence of a modified sequence of nucleic acid in transgenic food (Ehlers et al., 1997; Köppel et al., 1997; Stüder et al., 1997; Pietsch et al., 1997. One of these (Pietsch et al., 1997) has been validated by an interlaboratory trial in a group of European laboratories. More recently, a quantitative GMO detection method using competitive PCR has been described by Stüder et al. (1998), which is the most common method for quantitative PCR (Becker-Andre, 1991; Piatak et al., 1993).

Another quantitative PCR procedure, using the ABI Prism 7700 sequence detection system, has been shown to be extremely accurate and less labor-intensive (Desjardin et al., 1998; Heid et al., 1996; Livak, 1996). This procedure, better known as TaqMan (Holland et al., 1991), is based on the use of a fluorogenic probe that hybridizes within the target sequence bound by usual PCR primers. The probe is labeled with a fluorescent reporter dye on the 5′ end and with a fluorescent quencher dye on the 3′ end. Due to the closeness of the quencher to the reporter, the reporter fluorescence is suppressed. During PCR, the 5′ to 3′ exonuclease activity of Taq DNA polymerase degrades the hybridized probe and separates the two dyes. The resulting increase of fluorescence is proportional to the amount of specific PCR products.

In this paper, we describe the use of the ABI Prism 7700 device to quantify the amount of "Maximizer" transgenic maize and "Roundup Ready" transgenic soybean as a function of total maize or soybean, respectively, in food.

MATERIALS AND METHODS

Materials. Standard curves were calibrated with commercial transgenic soybean and maize reference standards (Fluka, Buchs, Switzerland). These standards are Certified Reference Material consisting of dried soybean and maize powders and were developed by the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) for the European interlaboratory trial mentioned above.

DNA Isolation. DNA was isolated from reference standards and foods using the procedure described by Köppel et al. (1997) with minor modifications. One gram of sample was incubated with 3 mL of TNE buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), and 1% (w/v) sodium dodecyl sulfate], 350 μL (5 M) of guanidine hydrochloride (Sigma, St. Louis, MO), and 140 μL (20 mg/mL) of protease from *Streptomyces griseus* (Sigma) at 50 °C for 2−15 h. The extracted DNA was purified using the Wizard protocol (Promega, Madison, WI). An additional step using Qiaquick spin columns (Qiagen, Hilden, Germany) was performed. DNA was eluted in 50 μL of EB buffer (Qiagen, Hilden, Germany) and then quantified spectrophotometrically with a Lambda Bio spectrophotometer (PE Applied Biosystems, Foster City, CA).

Oligonucleotide Primers and Probes. PCR systems (primers pair and fluorogenic probe) were designed using Primer Express software (PE Applied Biosystems) and purchased from PE Applied Biosystems. For both GMOs, two PCR systems were chosen (see Table 1), one for the total detection of maize or soybean (endogenous PCR system) and the other

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Table 1. Primer Pairs and Fluorogenic Probes Used in This Study

PCR system	orientation	name	sequence	GenBank or EMBL accession no.	position
endogenous maize	antisense primer	Zetm1	5′-TGTTAGGCGTCATCATCTGTGG-3′		250−271
	sense primer	Zetm3	5′-TGCAGCAACTGTTGGCCTTAC-3′	X07535	203−223
	sense probe	Zetmp	5′-ATCATCACTGGCATCGTCTGAAGCGG-3′		223−248
transgenic maize	sense primer	Crytm1	5′-GTGGACAGCCTGGACGAGAT-3′		1219−1238
	antisense primer	Crytm2	5′-TGCTGAAGCCACTGCGGAAC-3′	I41419	1305−1324
	sense probe	Crtmp	5′-AACAACAACGTGCCACCTCGACAGG-3′		1249−1273
endogenous soybean	sense primer	Sltm1	5′-AACCGGTAGCGTTGCCAG-3′		1253−1270
	antisense primer	Sltm2	5′-AGCCCATCTGCAAGCCTTT-3′	K00821	1315−1333
	sense probe	Sltmp	5′-TTCGCCGCTTCCTTCAACTTCACCT-3′		1272−1296
transgenic soybean	sense primer	Sttmf3a	5′-GCAAATCCTCTGGCCTTTCC-3′		99−118
	antisense primer	Sttmr2a	5′-CTTGCCCGTATTGATGACGTC-3′	I43998	224−244
	sense probe	Sttmpa	5′-TTCATGTTCGGCGGTCTCGCG-3′		164−184

for the specific detection of "Maximizer" maize or "Roundup Ready" soybean (transgenic PCR system). For maize, the endogenous PCR system amplified a part of *10-kDa zein* gene (Kirihara et al., 1988) and the transgenic PCR system targeted the *cryIA(b)* gene (Koziel et al., 1997). For soybean, PCR systems were developed on the *Le1 lectin* gene (endogenous; Vodkin et al., 1983) and on the CP4 *EPSPS* gene (transgenic; Barry et al., 1997). Endogenous probes were labeled with the fluorescent reporter dye tetrachloro-6-carboxyfluorescein (TET) on the 5′ end and transgenic probes with 6-carboxyfluorescein (FAM) on the 5′ end. The fluorescent quencher dye, 6-carboxy-tetramethylrhodamine (TAMRA), was located on the 3′ end of the probes.

During PCR cycling, the software calculates the emission of reporter and quencher dyes every 7 s. The ABI Prism 7700 is supplied with Sequence Detector software able to determine the contributions of each component dye spectra by means of a multicomponenting algorithm. The ΔRn values reflect the quantity of fluorescent probes degraded and fit an exponential function generating a real-time amplification plot for each well. The threshold is set at 10 times the standard deviation of the mean baseline emission calculated between the 3rd and 15th cycles. The point at which the amplification plot crosses the threshold is defined as Ct. Ct is reported as the fractional cycle number reflecting a positive result. The amount of DNA in an unknown sample is measured by interpolation from a standard curve of Ct values generated from known starting DNA concentrations. Total DNA quantity was determined by reporting the Ct TET value on the endogenous standard curve, and transgenic DNA quantity was fixed by reporting the Ct FAM value on the transgenic standard curve. Because both endogenous and transgenic PCR systems were performed in the same tube, a direct comparison between total and transgenic DNA was possible. The percentage of transgenic material was then determined as being the ratio of transgenic to total DNA quantities.

PCR conditions were then optimized to carry out maize endogenous and transgenic PCR systems in the same tube without any interference between one PCR reaction and the other one. The same strategy was used for soybean. Endogenous and transgenic primers or probes were designed to be used at the same hybridization temperature and $MgCl_2$ concentration. Furthermore, as we observed in transgenic low-content samples that transgenic PCR was restricted by the endogenous PCR, we intentionally limited the endogenous PCR by reducing the endogenous primer concentration in comparison with the transgenic primer concentration (see below). In these conditions, we checked that, on the one hand, the same endogenous Ct value was kept with or without limited endogenous primer concentration and that, on the other hand, endogenous and transgenic Ct values were the same if endogenous and transgenic PCRs were performed in only one or separate tubes (data not shown).

PCR Conditions. Amplification reactions (50 μL) were performed with the TaqMan PCR core reagents (Perkin-Elmer−Roche Molecular Systems, Branchburg, NJ). They contained 25 nM endogenous primers, 100 nM transgenic primers, 200 nM endogenous and transgenic probes, dATP, dCTP, and dGTP, each at a concentration of 400 μM, 800 μM dUTP, 2.5 units of AmpliTaqGold DNA polymerase, 0.5 unit of AmpErase uracil *N*-glycosylase (UNG), 6 and 6.5 mM $MgCl_2$ for soybean and maize PCR systems, respectively, and 1× TaqMan buffer A. Ten microliters of DNA (10 ng/μL) extracted from 0−2% of transgenic maize and soybean reference standards was used to determine the reproducibility of Ct measurements (see Table 2). For the generation of standard curves, stock DNA from the 2% transgenic maize or soybean was diluted to the following concentrations: 10, 5, 1, 0.5, 0.25, 0.1, and 0.05 ng/μL. Ten microliters was included in the amplification reaction, giving ranges from 100 to 0.5 ng and from 2 to 0.01 ng for endogenous and transgenic DNA quantities, respectively.

PCR reactions were run with the ABI Prism 7700 sequence detection system (PE Applied Biosystems) with the following program: 2 min at 50 °C, 10 min at 95 °C and 50 cycles, 15 s at 95 °C, and 1 min at 60 °C.

Table 2. Reproducibility of the Ct Measurements of Replicate Standards from 0 to 2% of Transgenic Content

quantity (% of transgenic content)	Ct for reaction 1	2	3	4	mean	SD[a]	% CV[b]
"Maximizer" Maize							
total (TET dye)							
0%	23.9	23.6	23.8	23.9	23.8	0.15	0.65
0.1%	24	23.9	24	23.7	23.9	0.14	0.61
0.5%	23.2	23.1	23.1	23.3	23.2	0.09	0.41
2%	24.1	23.9	23.6	23.9	23.9	0.23	0.97
transgenic (FAM dye)							
0.1%	33.3	33.3	33.3	33.5	33.4	0.1	0.3
0.5%	30.5	30.6	30.5	30.3	30.5	0.12	0.4
2%	28.7	28.1	28.2	28.6	28.4	0.28	1
"Roundup Ready" Soybean							
total (TET dye)							
0%	22.9	23.1	23.1	23.1	23.1	0.08	0.36
0.1%	22.5	22.7	22.7	22.3	22.6	0.16	0.73
0.5%	22.7	22.6	22.6	22.6	22.6	0.03	0.14
2%	23	22.9	22.6	22.6	22.8	0.21	0.93
transgenic (FAM dye)							
0.1%	35.3	35.2	35.1	34.9	35.1	0.15	0.43
0.5%	32.6	32.6	32.7	32.4	32.6	0.12	0.38
2%	30.3	30.3	30	30	30.1	0.2	0.65

[a] Standard deviation. [b] Coefficient of variation.

RESULTS AND DISCUSSION

Reproducibility of Quantification Assays. After optimization of primers, probes, and $MgCl_2$ concentrations (see above), we applied these PCR settings on known percentages of transgenic maize and soybean.

Reproducibility of the Ct measurements was then checked with a fixed amount of total DNA (100 ng) but with 0−2% of transgenic maize or soybean per sample (Table 2). For endogenous PCR systems, we obtained Ct values ranging from 23.1 to 24.1 cycles for maize and from 22.3 to 23.1 cycles for soybean. As expected, due

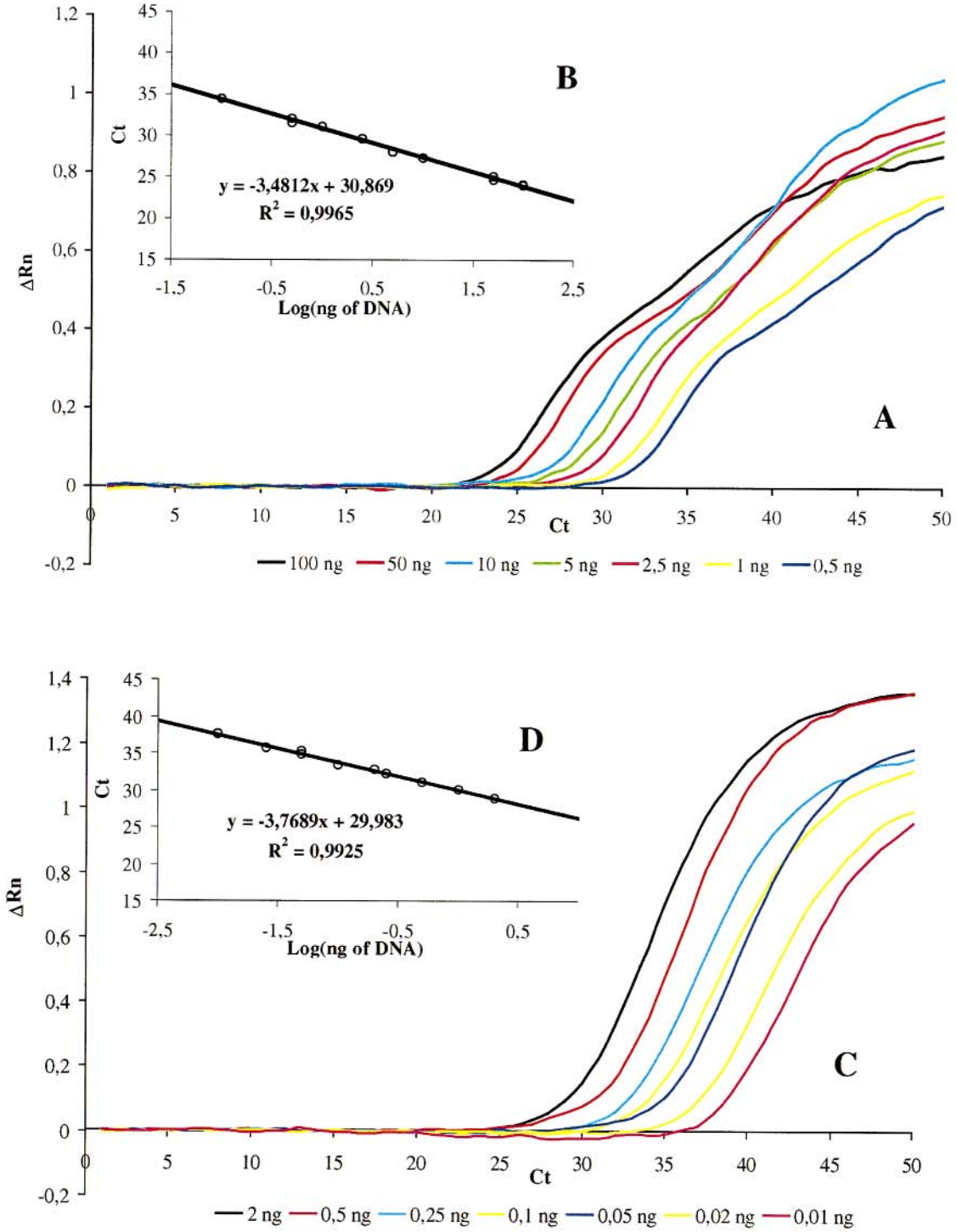

Figure 1. Maize PCR product detection in real time: (A) amplification plot generated by known amounts of DNA with endogenous PCR system (Zetm1, Zetm3, and Zetmp oligonucleotides); (B) standard curve from the data given in (A) (the equation and fit of the line are shown); (C) amplification plot generated by known amount of DNA with transgenic PCR system (Crytm1, Crytm2, and Crtmp oligonucleotides); (D) standard curve from the data given in (C) (the equation and fit of the line are shown).

to the fixed amount of DNA, Ct values did not vary with the transgenic material percentage. For the 16 values measured, we found coefficients of variation of 1.42 and 1.05% for maize and soybean, respectively. In the other case, transgenic PCR system Ct values were correlated with the amount of transgenic material percentage, varying from 28.1 to 33.5 cycles for maize and from 30 to 35.3 cycles for soybean, with coefficients of variation of a maximum of 1% in both cases. It clearly appeared that Ct measurements were highly reproducible, demonstrating the value in introducing real-time PCR in the quantitative detection of GMOs.

Generation of Standard Curves. The transgenic content of unknown samples may be easily quantified using a standard curve generated with a scale of diluted DNA from 0.1 to 2% of transgenic maize or soybean (Figures 1 and 2).

Because our calculation method is based on the ratio of transgenic to total DNA quantities, we were able to quantify 100% (if we found the same quantities of

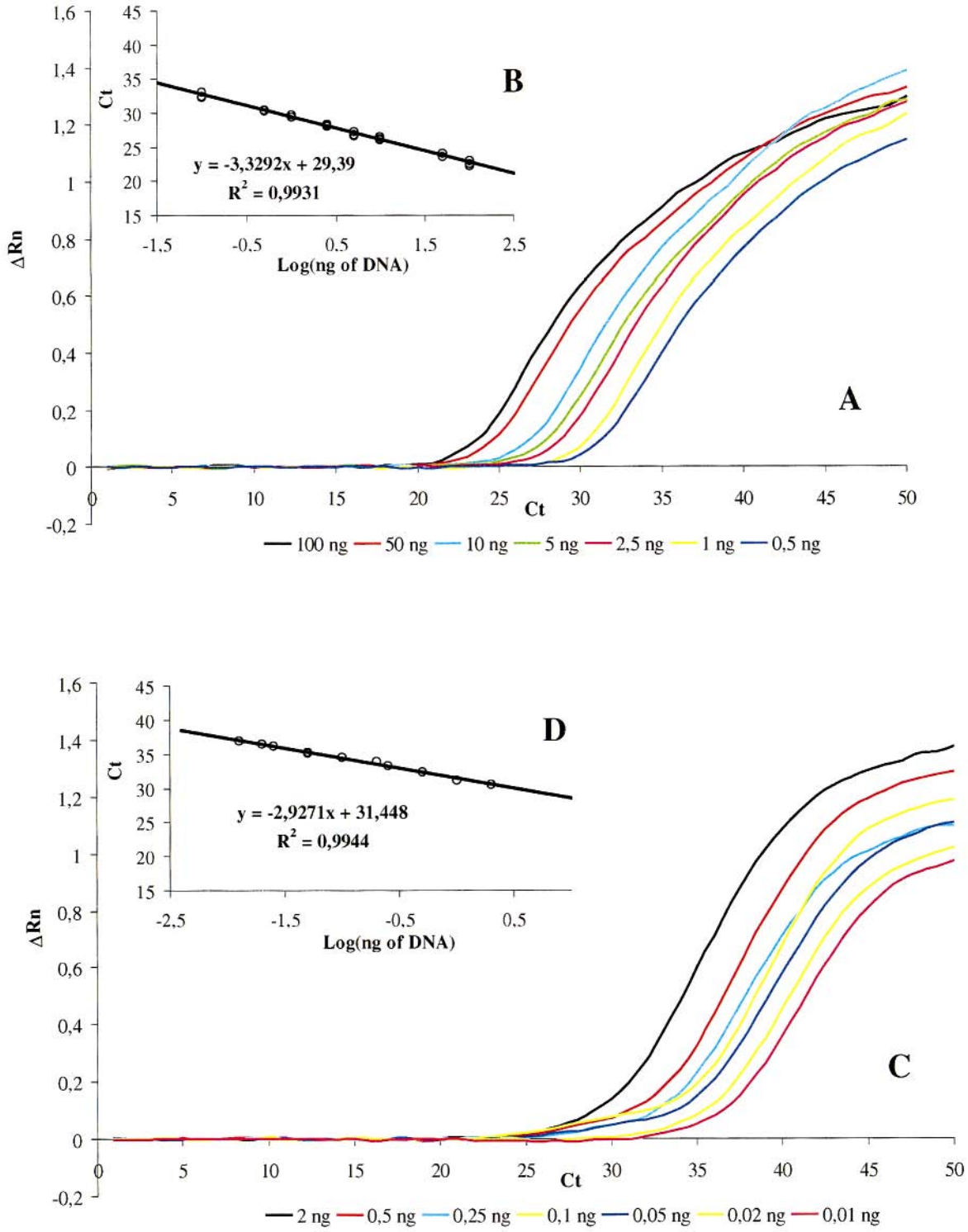

Figure 2. Soybean PCR product detection in real time. For details, see Figure 1 caption. Endogenous PCR system (Sltm1, Sltm2, and Sltmp oligonucleotides) and transgenic PCR system (Sttmf3a, Sttmr2a, and Sttmpa oligonucleotides) were used.

transgenic and total DNA) to 0.01% transgenic (if we found the highest total DNA quantity, i.e., 100 ng, and the lowest transgenic DNA quantity, i.e., 0.01 ng, of standards curves). The calculated R^2 values of standard curves were from 0.9925 to 0.9965. In the case of the endogenous standard curves, R^2 values were 0.9965 for maize and 0.9931 for soybean, which indicated again that the transgenic PCR system had no significant effect on the endogenous PCR system. Thus, real-time quantitative PCR allowed us to quantify GMOs over a very large range of starting DNA quantities. We were then able to detect at least 2 pg of either total or transgenic DNA per gram of starting sample. Referring to the genome sizes of maize and soybean (2.6 and 1.1 billion bases per haploid genome, respectively; Delseny and Glaszmann, 1995; Funke et al., 1993), we were theoretically at the unique genome copy level.

Quantitative Detection of Transgenic Maize and Soybean in Food Products. The transgenic content of four commercial food products (maize starch, muesli, lecithin, and soybean proteins) was quantified according to the method we developed. The results are presented

Table 3. Determination of Transgenic Content in Food Products[a]

sample	Ct	DNA (ng)	R^2	SD[a]	% CV[b]	*a*	*b*	% transgenic
			"Maximizer" Maize					
maize starch	TET: 27.4	19.04747	0.9910	0.1333	0.486	−4.531	29.673	
	FAM: 33.7	0.12982	0.9906	0.1636	0.486	−3.632	32.048	0.68
muesli	TET: 33.4	1.09115	0.9939	0.1869	0.559	−5.040	31.938	
	FAM: 42.7	0.00729	0.9933	0.4499	1.054	−4.776	33.631	0.67
			"Roundup Ready" Soybean					
lecithin	TET: 27	1.38735	0.9935	0.5435	2.013	−3.882	27.574	
	FAM: 32.2	0.04133	0.9945	0.1709	0.531	−4.419	26.065	2.98
soybean proteins	TET: 24.7	10.17964	0.9957	0.1255	0.508	−4.017	28.798	
	FAM: 30.3	0.11772	0.9943	0.1999	0.66	−4.301	26.353	1.16

[a] R^2 and *a* and *b* values of the regression equation Ct = *a* log(ng of DNA) + *b* for endogenous and transgenic DNA quantification are indicated.

in Table 3. Maize starch and muesli contained 0.68 and 0.67% "Maximizer" maize and lecithin and soybean proteins, 2.98 and 1.16% "Roundup Ready" soybean, respectively. Our quantification procedure was then easily and reliably applied to various food products even for samples with a low DNA quantity (muesli and lecithin).

CONCLUSION

We developed a new method to quantify the percentage of "Maximizer" maize and "Roundup Ready" soybean in food products.

Because both total and transgenic PCRs were performed in the same tube, no variations higher than the ones inherent in the Ct measurements were observed between different PCR reactions or DNA extractions of the same sample. In contrast, the quantification procedure during which total and transgenic PCRs are carried out in separate tubes could be greatly affected by random differences in the reaction conditions such as pipetting errors.

In comparison with the quantitative competitive PCR applied by Stüder et al. (1998), the real-time quantitative PCR is at least 10-fold more sensitive in GMOs detection because we were able to detect 2 pg of total or transgenic DNA per gram of starting sample. It is also less labor-intensive, more accurate, and does not require any post-PCR manipulations, allowing us to carry out the analysis in 3 h after DNA extraction. We routinely perform the whole procedure (DNA extraction and quantification) on a 24-sample set in under 12 h. Moreover, the use of dUTP and UNG in our PCR reactions eliminated carry-over contamination, which is the major source of false positives.

We successfully transposed this procedure to various raw materials, ingredients, and foods containing maize or soybean (seeds, flour, grits, pellets, cereal mix, starch, corn syrup, soybean proteins, lecithin, flavor, margarine, corn flakes, muesli, chocolate, fruit filling for biscuits, fruit preparation for dairy products, ketchup, cookies, brownies, dairy products, soft drinks, and beers) with the exception of oil, which needs a time-consuming DNA extraction protocol (Hellebrand et al., 1998). The procedure developed may require further validation on a wider range of corn products, which may possibly contain zein codons different from the region from which the probe is designed. This method can be used for the quantitative detection of other GMOs provided appropriate sequences are available for the choice of primers and probes.

We are currently working on the detection of other GMOs and on a mathematical modeling of the multiplex PCR allowing a more accurate quantification of the transgenic content.

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