

Detection of Genetically Modified Soy in Doughs and Cookies

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ABSTRACT

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In many countries, including the European Union member states, Switzerland, Australia, New Zealand, and Japan, legislation has been set up for labeling of genetically modified organisms (GMOs) in food and feed products. To comply with these regulations, reliable detection methods are necessary. If the detection is based on DNA, a GMO analysis may contain several steps where qualitative and quantitative species-specific, GMO screening, GMO construct, and GMO line-specific polymerase chain reactions (PCRs) are used. A limit of detection (LOD) thereby defines to what extent a target molecule may be detected in a sample. In this study, cookies were made with variable levels of a soy sample containing 2 wt%

Roundup Ready soy. For all PCRs described, detection limits based on dilution series and practical LODs were determined. The practical LODs are used to determine to what extent a GMO ingredient may be detected in a real food product. Results reveal that, due to the baking process, the overall DNA fragment length is reduced, rendering GMO analyses more difficult. Furthermore, Roundup Ready soy line-specific and real-time quantitative PCR are less sensitive than GMO screening PCRs, whereas just these PCRs are crucial in the decision-making process regarding the presence of GMOs in a food product. Moreover, high standard deviations and errors render the precise quantification of GMOs difficult.

Internationally, different legislations exist for the authorization and labeling of genetically modified organisms (GMOs) in food products. According to EU Regulations 1829/2003 (EC 2003a) and 1830/2003 (EC 2003b), the use of genetically modified soy should be mentioned on the label of the food product. This labeling may be omitted in cases where the GMO level is <0.9% of each ingredient, provided that it is a matter of accidental or technically unavoidable presence (EC Regulations 1829/2003 and 1830/2003). In other parts of the world, other threshold limits apply. For example, a 5% level of tolerance has been discussed for Japan and Taiwan; a threshold of 3% is used for South Korea; a 1% level is applied in New Zealand and Australia; Norway set a 2% threshold. Switzerland and the Russian Federation recently lowered their threshold to 0.9%. In the United States and Canada, labeling GM products is voluntary, not mandatory (Centre for Food Safety 2006).

Worldwide, GMO analyses are based on detection of the newly introduced DNA or expressed proteins. Especially in Europe, the technique used to check for the compliance with GMO regulations is the DNA-based polymerase chain reaction (PCR) method, which is well known for its high sensitivity and specificity (Holst-Jensen et al 2003). Nevertheless, compared with protein-based methods, equipment and operation are costly, and highly trained personnel are required (Wiseman 2002; Holst-Jensen et al 2003). Moreover, the acquisition of reliable results might be negatively influenced by the presence of PCR inhibitors and deterioration of DNA through the processing of the product (Anklam et al 2002). Reliable detection and quantification, however, also depend on the initial amount of target product present in the food product.

In this context, there are at least three different ways of expressing detection and quantification limits: absolute, relative, and practical limits (Berdal and Holst-Jensen 2001). The absolute limit is the lowest number of initial template copies that can be detected and quantified. Generally, detection and quantification limits are established through the PCR detection of a dilution series, with absolute detection limits being lower than quantification limits. To mimic real-life circumstances, nontarget DNA is added as background signal (Hupfer et al 1998; Jankiewicz et al 1999; Rønning et al 2003). These situations, however, still remain idealized. The

values of the absolute limits could depend on the chemistry of the PCR (Terry and Harris 2001) but in most cases described in GMO-related literature, an absolute limit of detection (LOD) as low as 1 initial template molecule can be obtained (Väitilingom et al 1999; Berdal and Holst-Jensen 2001; Terry and Harris 2001; Hernández et al 2003; Nielsen et al 2004), while the absolute limit of quantification (LOQ) is 5–40 initial template copies (Berdal and Holst-Jensen 2001; Alary et al 2002; Rønning et al 2003; Nielsen et al 2004). Below these values, the probability that repetitions contain relevant DNA copies is insufficiently high. Therefore, to make sure that at least one copy of Roundup Ready soy is present in the PCR, the expected number of included copies should be slightly higher than 1. The same holds for the absolute quantification limits (Berdal and Holst-Jensen 2001). The relative limit refers to the lowest percentage of GMOs (i.e., the relative percentage of initial copies of the transgene to the total amount of copies of the reference gene) that can be quantified. The practical limit is the functional limit of the sample during an analysis (Jankiewicz et al 1999; Berdal and Holst-Jensen 2001).

In this research, practical detection and quantification limits were determined. Cookies were made with different amounts of a soybean sample containing 2 wt% Roundup Ready soy (RRS). The practical detection limits of a soy-specific PCR, GMO screening PCRs, a Roundup Ready soy construct, and line-specific PCRs, as well as quantification limits of a real-time quantitative PCR were calculated. Values are expressed as the actual amount of RRS that should be added to a cookie to guarantee reliable GMO detection and quantification. Furthermore, to examine the influence of the baking process on the DNA integrity and detection through PCR, the doughs were analyzed as well.

MATERIALS AND METHODS

Preparation of Cookies

For the preparation of the cookies, Approved Method 10-50D (AACC International 2000) was used. Dough (472.6 g) was made with 225.0 g of wheat flour, 64.0 g of margarine, 130.0 g of sugar, 33.0 g of a 5.9% (w/v) dextrose solution, 2.5 g of sodium bicarbonate, 2.1 g of salt, and 16.0 g of water were used. Before baking, the dough was rolled out to a thickness of 6.2 mm, cut out in slices of 63.5 mm, and then baked for 10 min at 205°C.

Extraction of Genomic DNA

DNA was extracted from the doughs and cookies by a CTAB method (ISO 21571). DNA integrity was checked by loading 10 µL of extract on a 2% agarose gel stained by ethidium bromide and the DNA concentration was checked by spectrophotometry

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(Genequant PRO, Amersham Pharmacia Biotech, Roosendaal, The Netherlands). For the spectrophotometrical analysis, samples were diluted 1/20. The DNA concentration and impurity factor (absorbance at 260 nm/absorbance at 280 nm) were recorded.

Oligonucleotide Primers

The primers GMO3 (5'-GCC CTC TAC TCC ACC CCC ATC C-3') and GMO4 (5'-GCC CAT CTG CAA GCC TTT TTG TG-3') are specific for the detection of the single-copy lectin gene *le1* and yield a PCR product of 118 bp (Meyer et al 1996). The lectin gene is present in transgenic as well as in conventional soybeans.

The next step in the GMO analysis involves screening samples for the presence of genes that are specific for regulatory sequences. As these sequences are present in nearly all the currently marketed GMOs, a PCR targeting these sequences is used as an indication of genetic manipulation. A first-screening PCR targets the 35S DNA sequence, derived from Cauliflower Mosaic Virus (CaMV), which is introduced into GMOs to promote the expression of the inserted gene. Primers p35S-cf3 (5'-CCA CGT CTT CAA AGC AAG TGG-3') and p35S-cr4 (5'-TTC TCT CCA AAT GAA ATG AAC TTC C-3') amplify a PCR fragment of 123 bp. A second screening PCR searches for the presence of 3'-*nos* DNA sequence, a nopaline synthase gene derived from the soil bacterium *Agrobacterium tumefaciens*, which is used in GMOs to end the expression of the newly introduced gene. Primer pair HA-*nos*118-f (5'-GCA TGA CGT TAT TTA TGA GAT GGG-3') and HA-*nos*118-r (5'-GAC ACC GCG CGC GAT AAT TTA TCC-3') amplify a sequence of 118 bp (Lipp et al 2001).

Nearly all approved GMOs used in food contain one or both of these regulatory sequences. In Roundup Ready soy, both the 35S and the 3'-*nos* DNA sequences are present.

The primers 35s-f2 (also: B1) (5'-TGA TGT GAT ATC TCC ACT GAC G-3') and petu-r1 (also: B2) (5'-TGT ATC CCT TGA GCC ATG TTG T-3') are specific for detecting the newly introduced construct in Roundup Ready soy. These construct-specific primers attach to the CaMV 35S promoter sequence and to the petunia hybrid chloroplast transit-signal sequence, respectively, resulting in a 172 bp fragment (Jankiewicz et al 1999). However,

two GMO events cannot be distinguished by the application of construct-specific methods presently included in the European and international standard (ISO 21569, ISO 21570, Holst-Jensen et al 2006).

A true identification of Roundup Ready soy is achieved with event- or line-specific primers that target the junction between the newly introduced genes and the integration site. For the RRS event-specific detection, primer pair *nosRR2* (5'-GCG CGG TGT CAT CTA TGT TA-3') and *nosRR4* (5'-AGG TGT CGC CTT CCT TAC G-3') were used, resulting in a 177 bp amplicon (Taverniers et al 2001).

Except for the event-specific detection, all primers form part of the international ISO 21569 norm for the qualitative detection of GMOs. The location of the primer pairs on the Roundup Ready soybean genome are depicted in Fig. 1.

Amplification of Soy DNA Fragments

For GMO3/4, B1/B2, and *nosRR2/4* reactions, the PCR mix including 2 µL of extracted DNA product was added to 36.6 µL of bidistilled water, 5 µL of 10× GeneAmp PCR buffer I (Applied Biosystems, Lennik, Belgium), 2 µL of each primer (10 µM), 2 µL of dNTP solution (5 mM), and 0.4 µL of *AmpliTaq* DNA polymerase (5 U/µL, Applied Biosystems). For 35S-CF3/CR4 and HA-*nos*118F/R reactions, 5 µL of a 1/5 dilution of the DNA sample solution was added to a PCR mix containing 12.04 µL of bidistilled water, 2.5 µL of 10× GeneAmp PCR buffer II (Applied Biosystems), 1.5 µL of a 25 mM MgCl₂ solution (Applied Biosystems), 1.5 µL of primer 1 (10 µM), 1.5 µL of primer 2 (10 µM), 0.8 µL of dNTP solution (5 mM), and 0.16 µL of *AmpliTaq* Gold polymerase (5 U/µL, Applied Biosystems).

Table I lists the PCR thermal profiles applied for different primer pairs using a GeneAmp PCR 9700 system (Applied Biosystems).

The amplification of the lectin gene was performed according to Gryson et al (2002). DNA fragments were separated by gel electrophoresis in a 2 % agarose gel with TAE buffer, stained with ethidium bromide, and visualized using the ImageMaster VDS UV transilluminator system of Amersham Pharmacia Biotech (Roosendaal, The Netherlands).

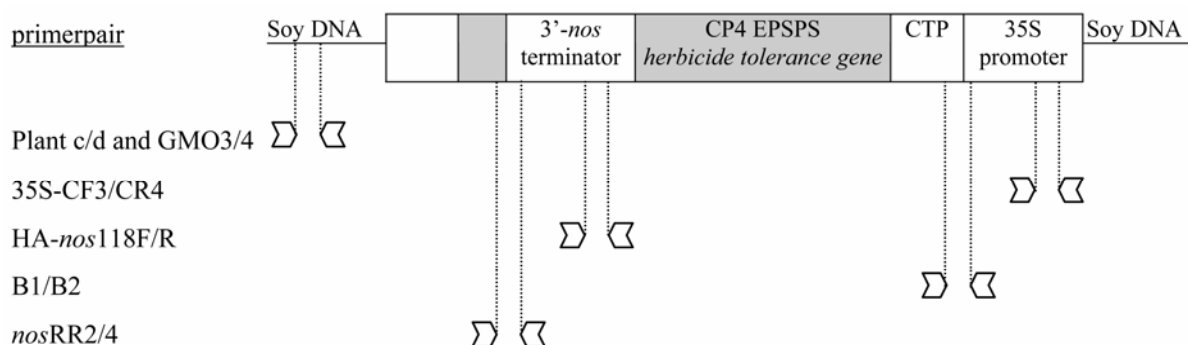


Fig. 1. Amplified fragments of the Roundup Ready soybean genome (not drawn in scale). From right to left: 35S promoter, chloroplast transit peptide (CTP); CP4 EPSPS coding sequence (gray); 3'-*nos* terminator, an additional small part of the CP4 EPSPS (gray); and a rearrangement DNA segment (Windels et al 2001).

TABLE I
PCR Thermal Profiles Applied to Different Primer Pairs^a

Primer Pair	GMO3/4	35S-CF3/CR4	HA- <i>nos</i> 118F/R	B1/B2	<i>nosRR2/4</i>
Initial denaturation	5 min 95°C	10 min 95°C		3 min 95°C	3 min 95°C
Denaturation	30 sec 95°C	25 sec 95°C		25 sec 95°C	20 sec 95°C
Annealing	30 sec 60°C	30 sec 62°C		30 sec 62°C	40 sec 54°C
Extension	30 sec 72°C	45 sec 72°C		45 sec 72°C	30 sec 72°C
Final extension	3 min 72°C	7 min 72°C		7 min 72°C	3 min 72°C
Cycles	35	50		35	35

^a Polymerase chain reactions (PCRs); genetically modified organisms (GMOs).

GMO Quantification

For the quantification of the Roundup Ready soy content through real-time PCR, the GMOQuant Roundup Ready soy DNA Quantification System 7700 kit from GeneScan (Freiburg, Germany) was used. The kit contains Roundup Ready soy and soy reference master mixes, calibration DNA standards, and one tube of certified reference material (CRM), which is a soy sample containing 0.5 wt% Roundup Ready soy, as a positive control. For analysis, samples were diluted 1/10, as recommended by the producer, except for the positive control. A volume of 5 μ L of this diluted DNA solution was added to 20 μ L of master mix. The thermocycler program settings, using a ABI Prism 7000 Sequence Detection System (Applied Biosystems, Lennik, Belgium) were 1) 10 min at 95°C; and 2) 15 sec at 95°C and 1 min at 60°C with 45 repeats. Duplex reactions were performed with the standard DNA series, samples, and positive control (CRM).

Determination of Absolute Detection Limit

For the determination of the absolute detection limit, DNA from a soybean flour sample containing 2 wt% Roundup Ready soy (RRS) material, was extracted and the concentration of this DNA extract was determined with a UV spectrophotometer (Bio-photometer, Eppendorf, Hamburg, Germany). Spectrophotometrically, an absorption of one unit at a wavelength of 260 nm (in the UV region) equals 50 ng/mL of dsDNA. The Roundup Ready soybean sample was then used to set up a 10-fold dilution series till a dilution of 10^{-5} . PCR were performed 10 times on each dilution.

The 2 wt% ground and blended Roundup Ready soybean sample was obtained through the participation in ring trials organized by AACC International. This sample will be further referred to as the 2 wt% Roundup Ready soy (RRS) containing soy sample.

Determination of Practical Detection Limit

For the calculation of the practical detection limit, part of the wheat flour was exchanged with a sample containing 2 wt% RRS to variable extents (0.01, 0.05, 0.1, 0.5, 1.0, 1.2, 1.5, 2.0, 5.0, 10.0, and 15 g).

RESULTS

Influence of Baking Process on DNA Integrity

The effect of heat exposure on the DNA in a cookie dough was examined through the comparison of the integrity of DNA before and after baking. Variations in the DNA fragment length, a parameter for DNA integrity, are dependent on the material under examination, the degree of processing the sample has been subjected to, and the DNA extraction method applied. For this purpose, DNA integrity was examined by loading some of the extracted DNA on an agarose gel before PCR amplification. The DNA extract from some of the analyzed doughs (Fig. 2A) showed an intense smear of short and long DNA fragments, with a high concentration of DNA fragments $>2,000$ bp (highest band of the DNA marker is 2,072 bp). Due to the baking process, the intensity of the DNA smears was significantly decreased (Fig. 2B). The high concen-

tration of long DNA fragments was totally lost due to the heat exposure of 205°C over 10 min. Nevertheless, DNA fragments of all ranges of length are still present, presuming that the amplification of DNA through PCR should still be possible. These results show great resemblance to the findings of Straub et al (1999), who investigated the baking of bread. DNA purity and quantity were checked through the spectrophotometrical analysis of some of the DNA from the dough and cookie samples. The impurity factor is defined as the proportion of the absorbance at 260 nm and at 280 nm. For doughs, an overall mean value for impurity was 1.73, whereas for the cookies an overall mean value for impurity was 1.72. These high values prove that DNA extracts are pure. Few contaminants in terms of proteins are present, which might interfere with the PCR amplification.

Although agarose gel electrophoresis showed a loss of DNA through baking, these differences could not be observed based on DNA concentrations calculated with spectrophotometry. Overall mean DNA concentration for the doughs was 180 ng/ μ L \pm 31 and 206 ng/ μ L \pm 42 for the cookies. For some cookies, an increase in DNA concentration could be noticed compared with the corresponding dough, whereas the opposite could also be observed.

Determination of Absolute and Practical Detection Limits

Absolute detection limit. For the determination of the absolute detection limit (LOD_{ABS}) of the different PCRs, DNA from a 2 wt% RRS sample obtained from an AACC International ring trial was extracted and a 10-fold dilution series was set up. The concentration of the extracted and undiluted DNA was determined spectrophotometrically to be 2,356 μ g of DNA/mL. Table II shows the results for the soy-specific GMO screening, RRS construct, and line-specific PCR detection on the dilution series (10^0 to 10^5) of the DNA from the 2 wt% RRS sample. PCRs were performed

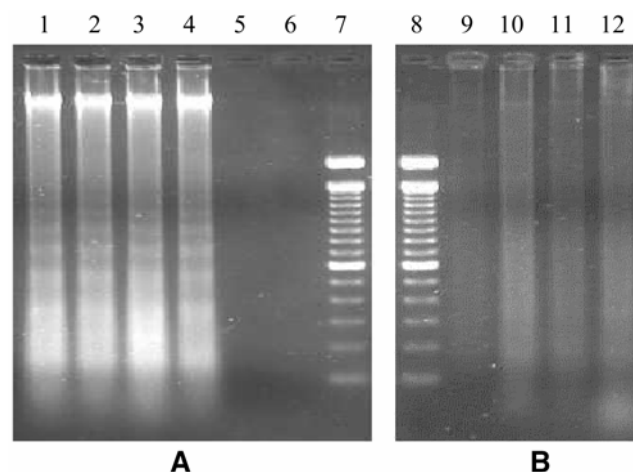


Fig. 2. Integrity of DNA extracted from (A) cookie dough and (B) baked cookie. Lanes 1–4: DNA from cookie dough; lanes 5 and 6: extraction blanks; lanes 7 and 8: 100 bp DNA ladder; lanes 9–12: DNA from baked cookie.

TABLE II
Amplification Results to Determine LOD_{ABS} of Several Primer Pairs with a 10-Fold Dilution Series of a Soybean Sample Containing 2 wt% Roundup Ready Soy^a

Primer Pair	Dilution						LOD_{ABS} (target copies)
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
GMO3/4	10/10	10/10	10/10	10/10	7/10	0/10	408
35S CF3/CR4	10/10	10/10	10/10	10/10	8/10	0/10	204
HA-nos118F/R	10/10	10/10	10/10	10/10	9/10	1/10	204
B1/B2	10/10	10/10	10/10	6/10	0/10	0/10	816
nosRR2/RR4	10/10	10/10	10/10	4/10	0/10	0/10	816

^a Limit of absolute detection (LOD_{ABS}), polymerase chain reactions (PCRs); genetically modified organisms (GMOs). Positive results of 10 replicates.

10 times and positive amplification results (where a clear amplification product could be visualized at the height of the positive PCR control on the agarose gel) were counted for the determination of the detection limit.

If the absolute detection limit is considered as the lowest concentration where detection of all 10 replicates is still possible, the detection limits are quite high (Table II). Moreover, if the detection limit is determined at a 100% repeatability, the information regarding the next dilution step is lost. Therefore, the question was raised how many positive signals out of 10 should be needed to consider that dilution as positive, with an acceptably high accuracy. To do so, the criterion to consider a dilution as positive was set at a minimum 7 positive signals out of 10; acceptable accuracy was set at a minimum 95%. The probability to reject a dilution series with 7/10 positive signals, while this dilution should have been considered as positive, is then 0.03%. As this chance is very low, the choice to consider at least 7 positive signals out of 10 to determine the detection limit is satisfactory.

Results in Table II show that for the GMO screening PCRs and the species-specific PCR, the detection limit is reached at the 10^{-4} dilution. However, when comparing the results of different PCRs, the number of PCR cycles, and the amount of target DNA should also be taken into account. Therefore, the number of initial target DNA copies present in every PCR was determined. For these calculations, a length of 1,115 Mbp for the haploid genome of soybean and a conversion of 1 pg of DNA to 965.10^6 bp was assumed (Aramuganathan and Earle 1991). Results in Table II show that the most sensitive PCRs clearly are the GMO screening PCRs. The species-specific PCR is less sensitive, whereas the RRS construct and line-specific PCRs are the least sensitive.

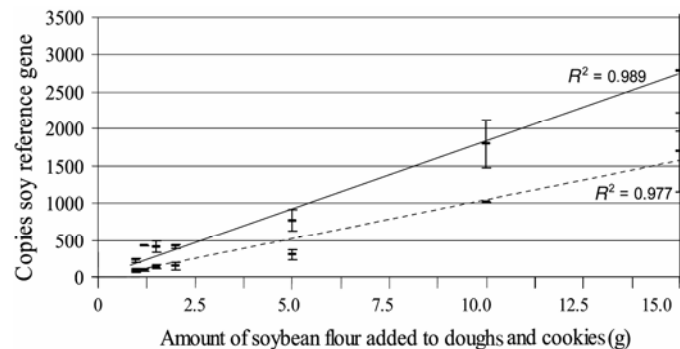


Fig. 3. Number of copies of soy reference gene detected in relation to the amount of soybean flour added to a dough (solid line) and baked cookie (dotted line).

Practical detection limit. For the determination of the practical detection limit (LOD_{PRAC}), the 2 wt% RRS containing soy sample was mixed in the dough in a concentration ranging from 0 to 3.2 wt% on a wet weight basis. The dough was then baked to evaluate the influence of the baking process on GMO detection. DNA was extracted in duplex from dough and cookies, after which the different PCR analyses were performed. This experiment was then repeated to obtain four amplification results for each dilution. For the doughs as well as for the cookies, a 100% positive amplification was observed for the species-specific PCR detection when 0.100 g of RRS or more was added. This corresponds to a detection limit of 0.02 wt% soy. However, the amplification signals for the cookies were clearly less intense than for the doughs, due to the baking process.

The results for the other PCRs, expressed as the amount of a 2 wt% RRS sample needed in a dough or cookie to obtain a positive amplification of the four replicates are summarized in Table III. Again, screening PCRs are the most sensitive. The highest practical limit of detection is obtained for the RRS event-specific PCR with primer pair *nosRR2/4*, followed by the RRS construct-specific PCR with primer pair B1/B2. This is an important result because the first step in a GMO analysis is generally a test for amplification with species-specific primers. If this result is negative, the GMO analysis is usually terminated at this point.

GM Quantification

For the quantification of RRS in the doughs and cookies, the GMOQuant Roundup Ready soy DNA Quantification System 7700 kit from GeneScan (Freiburg, Germany) was used. Slopes of the standard curves had a mean value of -3.23 ± 0.30 . Correlation coefficients of the standard curves were >0.98 . Results in Fig. 3 show the relationship between the amount of 2 wt% RRS flour sample added to a dough and the respective cookie and amount of soy reference genes detected. An increasing amount of soybean flour in the sample results in an increased detection of the soy reference gene, with a correlation coefficient of 0.989 for the doughs (full line) and an $R^2 = 0.977$ for the cookies (dotted line).

Statistically, the quantification limit (LOQ) is defined as the amount of target molecules that is still quantifiable with an acceptable precision and correctness. The objective of an optimized PCR system is to detect one molecule but this varies from primer pair to primer pair. According to the manufacturer's manual, a minimum of 80 copies for the reference gene and 10 copies for the RRS specific gene were set as absolute quantification limits.

The lowest amount of soy reference gene that could be detected for the samples was when 1.00 g of soy was added, for the dough as well as for the cookie (Fig. 3). That is at a 0.21 wt% level for the dough and a 0.24 wt% level for the cookie. However, through

TABLE III
Amplification Results to Determine LOD_{PRAC} of Several Primer Pairs with a Soybean Sample Containing 2 wt% Roundup Ready Soy Mixed in Doughs and Cookies^a

Primer Pair	Amount of GM Soy Added (mg)							LOD_{PRAC} (wt%)
	1,200	1,000	500	200	100	50	10	
Doughs (476.2 g)								
GMO3/4	4/4	4/4	4/4	4/4	4/4	2/4	0/4	0.02
35S CF3/CR4	4/4	4/4	4/4	4/4	0/4	0/4	0/4	0.04
HA- <i>nos118F/R</i>	4/4	4/4	4/4	4/4	0/4	0/4	0/4	0.04
B1/B2	4/4	4/4	4/4	0/4	0/4	0/4	0/4	0.11
<i>nosRR2/RR4</i>	4/4	4/4	0/4	0/4	0/4	0/4	0/4	0.21
Cookies (411.2 g)								
GMO3/4	4/4	4/4	4/4	4/4	4/4	0/4	0/4	0.02
35S CF3/CR4	4/4	4/4	4/4	0/4	0/4	0/4	0/4	0.12
HA- <i>nos118F/R</i>	4/4	4/4	4/4	0/4	0/4	0/4	0/4	0.12
B1/B2	4/4	4/4	0/4	0/4	0/4	0/4	0/4	0.24
<i>nosRR2/RR4</i>	4/4	0/4	0/4	0/4	0/4	0/4	0/4	0.29

^a Limit of practical detection (LOD_{PRAC}); polymerase chain reactions (PCRs); genetically modified organisms (GMOs).

extrapolation outside the measuring range (i.e., below LOQ of 80 copies), the soy reference gene could still be detected at a level of 0.1 g (0.02 wt%) for the dough (3 copies detected) and 0.2 g (0.05 wt%) for the cookie (2 copies detected).

Nevertheless, the best method is to calculate the LOQ in every sample because the LOQ is dependent on the amount of DNA extracted from the sample. This value is calculated with 10 copies of GMO-DNA being the total amount of conventional DNA found in the sample (equal to the reference DNA of the plant species in question, determined with a species-specific PCR).

In the dough, where 231 copies of the soy reference gene were detected at the 1.00 g level of RRS added, the LOQ was 4.4%. For the cookies, the LOQ for the 1.00 g soy sample was 10.7%. These values represent the amount of 2 wt% RRS that should be present in those samples to guarantee a reliable quantification. These calculations, however, assume that the detection of the soy reference gene is a limiting factor, not the RRS detection. In the context of the 0.9% labeling threshold value, 1,111 copies of the lectin gene are needed to enable a reliable GMO quantification. This would be attained when 2.5 wt% RRS is added to a cookie.

For the detection of the RRS-specific gene, a figure similar to that depicted in Fig. 3 could be drawn (data not shown). Due to the low amounts of the RRS in the samples, correlation between the amount of RRS added to the samples and the detection thereof through real-time PCR was low. An R^2 of 0.957 was obtained within the dough and an $R^2 = 0.963$ for the cookie. Due to the low number of copies of the transgene in the samples, the observed variation is higher and the correlation is lower.

The lowest amount of RRS-specific gene copies that could be detected was obtained at 5.0 g (1.1 wt%) for the dough (detection of 13 copies) and 10.0 g (2.4 wt%) for the cookie (detection of 12 copies). This means that a reliable GMO quantification can only be made at these levels. Through extrapolation, a limit of 10 copies is reached when 3.2 g of 2 wt% RRS sample is added to the dough (0.7 wt%). For the cookie, an amount of 7.4 g (1.8 wt%) should be added. A similar reasoning can be made for a sample containing 0.9% RRS, as this is the threshold level for labeling in case of accidental contamination. Extrapolation of results proves that 7.1 g (1.5 wt%) should be necessary in a dough and 16.4 g (4.0 wt%) in the cookie.

The results for the calculation of the true GMO content of the samples are given in Table IV. The observed quantitative estimates deviated up to 24% from the true value. The 2 wt% RRS sample used to prepare the food samples gave a GMO content of 1.55%. This is an error of 23%.

DISCUSSION

Generally speaking, the determination of an absolute detection limit, where a serial dilution of a DNA extract is used, is quite robust and is based on a one-ingredient food sample. Moreover, the calculation of the LOD_{ABS} is based on spectrophotometrical analysis, assuming that the total DNA content can be attributed to entire genome copies. The method is also prone to pipetting and dilution errors, and the use of 10-fold dilutions is merely a rough estimate. The use of a LOD_{PRAC} is based on real-life food prod-

ucts that contain several ingredients. Therefore, it gives a better image regarding the detection of the target DNA molecule, as it is expressed in terms of true addition of the ingredient under investigation. The LOD_{PRAC} is of much higher value for food processors and governmental inspection agencies.

According to the results summarized in Tables II and III, the detection limit for the GMO screening PCRs are the lowest of all PCR reactions. This can easily be explained by the fact that the screening for the 35S and the 3'-nos DNA sequences is based on a program of 50 PCR cycles, while only 35 cycles are needed for the other PCRs. When these GMO screening results are positive and the specific GMOs can be identified, a subsequent GMO quantification can be performed. However, due to the higher sensitivity of the GMO screening PCR, situations may be encountered where a positive signal is obtained for the GMO screening PCR but a negative result for the species-specific, line or construct-specific PCR. This is the case for the sample where 0.5 g of soy was added. Results in Table III show that although GMO screening is positive, at this level, the RRS specific detection results are negative, and the GMO quantification is unfeasible. Although the applied methods form part of an international standard (ISO 21569), caution should be taken during analyses as wrong conclusions might be drawn regarding the true GMO content of a food product.

More than with the qualitative PCR, which is an end-point measurement, differences in duplex measurements were observed with the real-time PCR quantifications. As a result, the determination of the GMO content may differ from the true value. These differences may be caused by pipetting errors, the use of separate tubes for the real-time PCRs, and differences in extraction efficiencies that are due to the nature of the enzymatic reactions involved (lysis of cells). These errors result in random differences in the reaction conditions. Moreover, the many manipulations during the CTAB extraction require a highly trained staff, and the extraction of many samples at the same time might result in altered extraction efficiencies between samples of the same extraction run. As quantification is a real-time PCR process, differences in duplex measurements are observed easily, while qualitative PCR is an end-point measurement, where differences in initial DNA concentrations are not necessarily noticed when loaded on a agarose gel. Due to these variations, the standard deviations of the GMO percentages of the samples were relatively high (10–40%).

Results for the calculation of the GMO percentage also clearly show that, except for one sample, all calculations were less than the predicted 2%. Errors range from 6 to 24%. Although these errors seem to be quite high, similar errors have been observed in other research (Rønning et al 2003). Moreover, it should be stated clearly that the soybean flour sample was prepared by mixing RRS with conventional soy on a w/w ratio and not on a genome/genome ratio. So differences in the genome/weight ratios of the two soybean materials (GMO and non-GMO) used to prepare the food samples might explain the discrepancy. The fact that most of the quantitative estimates are lower than the true value also can be partly explained by the characteristic of the PCR itself, which does not amplify a target sequence at a 100% efficacy. A decrease in efficiency will lead to an underestimation of the target copy number.

TABLE IV
Determination of Roundup Ready Soy Percentage in GMO/non-GMO in Doughs and Cookies Through Real-Time PCR^a

Soy Added	Dough		Cookie	
	% GMO/non-GMO	Trueness (error)	% GMO/non-GMO	Trueness (error)
5.0 g	1.69 ± 0.11	16%	2.12 ± 0.23	6%
10.0 g	1.54 ± 0.16	23%	1.52 ± 0.28	24%
15.0 g	1.71 ± 0.40	15%	1.71 ± 0.39	15%
0.5 wt% CRM	0.40 ± 0.17	20%	0.62 ± 0.11	24%

^a Genetically modified organisms (GMOs); polymerase chain reactions (PCRs); certified reference material (CRM).

Another explanation for the discrepancy between the experimentally determined and the theoretical values could be the sample preparation and the quality of the DNA extracts. Spectrophotometrical analysis of the samples, however, revealed relatively high A260/A280 ratios, presuming that DNA extracts were quite pure.

Although the lowest measuring range for the real-time detection of RRS is much lower than the measuring range for the soy reference (10 copies vs. 80 copies), the results clearly show that the quantification of the transgene is limiting. This is due to the low GMO content. With the 2 wt% RRS sample used, an amount of 4.0 wt% should be included in the cookie to allow a reliable quantification. For determination of lower GMO contents (GMO threshold level of 0.9% according to the 1829/2003 EC Regulation), it will be necessary to increase the amount of total soy DNA in the sample. Sensitivity could be increased if the amount of total DNA in the PCRs were increased. But this will also result in an increase of the amount of PCR inhibitors that may be present in the extracts. In fact, to quantify very low adulteration by real-time PCR, an equilibrium should be found between these two negatively correlated parameters.

With respect to the influence of the baking process on the detection of GMOs in cookies, results show that qualitative as well as quantitative detection are sensitive to the baking process. Taking the results of the spectrophotometrical analysis into account, where no significant decrease in the DNA concentration could be observed due to baking, it can be concluded that the baking process reduces the overall DNA fragment length. Taking the determination of the 35S or 3'-nos DNA sequences as the starting point of the GMO analysis, the possibility of performing a GMO analysis on a cookie is reduced by a factor two, compared with unprocessed dough.

CONCLUSIONS

It is well known that the PCR detection and quantification of GMOs depends on the quality, quantity, and purity of the DNA extracted. Therefore, the determination of the GMO content of food products is not an easy assignment. The processing of the food product may lead to decrease of the overall DNA fragment length. Moreover, the presence of ingredients other than the target DNA may interfere in the GMO detection process. In the baking of cookies, results have shown that the influence of processing is not great, and detection of GMOs remains possible to a certain extent.

Screening PCRs are the most sensitive PCRs in a GMO analysis. This means that situations may arise where positive screening results may not be confirmed by specific GMO identification or quantification PCR. Moreover, the quantification of GMOs is characterized by high errors. Standard deviations of duplex measurements may be high and GMO percentages may deviate from the true value. Therefore, focus in the future should at least be put on the quantification of the GMOs and reference genes in the same test tube (multiplexing).

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