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Quantitative analysis of genetically modified organisms (GMO) in processed food by PCR-based methods

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Abstract

Two different PCR-based approaches for the quantitative analysis of genetically modified organism (GMO) – components in foods are presented using Soybean derived samples as an example. The first method – a double competitive PCR – is well suited to determine threshold levels of GMO content in food. The other – PCR on-line measurement – is suited to determine ratios of transgenic versus non-transgenic component. Both methods provide a means to alleviate the problems of standardisation encountered with simple qualitative PCR approaches and will allow to cope with threshold levels for GMO, once issued by legislative bodies. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The most important prerequisites for the application of PCR-based detection methods are complete knowledge on the foreign genetic construct within the genetically modified organism (GMO) to be detected and the ability to extract significant amounts of amplifiable DNA from the sample to be investigated. Provided these two prerequisites are fulfilled, PCR-based detection methods can rapidly be designed for virtually any GMO within short of time. Generally this holds true also for quantitative approaches although more time is needed for development and validation. In fact availability of suitable reference material and criteria for standardisation rather constitute a bottle-neck at present.

So far, only qualitative methods for GMO detection have been standardised. But still inter-laboratory variability is substantial as variations in DNA-extraction efficiency and inhibitory effects can occur, thus needing careful monitoring.

Quantitative methods for the analysis of pure raw materials using competitive PCR have recently been developed and evaluated (see contribution of P. Hübner). They are based on the quantification of the DNA submitted to PCR (e.g., by photometric means) and subsequent measurement of the transgene specific DNA content using competitive PCR. These methods decrease inter-laboratory variation dramatically and the problem of false negatives due to inhibitory effects is eliminated by defining sensitivity of PCR with the internal competitor. One remaining problem is, that the quality of DNA (fragmentation or chemical modification) and the extent of contained non-chromosomal DNA (tissue-specific) cannot be addressed by these means. This makes it necessary to standardise the method for each single food matrix separately. Furthermore only pure matrices (derived from one organism) can be analysed (in terms of relative GMO content).

In general with processed food it is more difficult to obtain significant amounts of amplifiable DNA as heat treatment, enzymatic activities or acidic pH lead to fragmentation and other modifications of DNA (see also Hupfer et al., 1998).

In addition, the analysis of mixed samples or end products needs to determine relative proportions of GMO with respect to a certain food component e.g., the proportion of Roundup Ready Soy (RRS) in the whole soybean fraction within the sample. To determine this ratio in the example of RRS, the RRS specific DNA and the general soy specific DNA (as a reference) has to be determined, thus allowing a ratio to be calculated.

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The two methods presented below, make use of the soybean lectin le1 gene as a general soybean gene, that has already been exploited for other food related issues (e.g., Mayer et al., 1996).

The first method uses a double competitive PCR approach in which the RRS DNA and the general soy DNA content are measured by two independent competitive PCR's. The second is a quantitative approach using on-line PCR measurement using the so-called TaqMan technique on a Abi-Prism 7700 device (Anonymous, 1997); alternatively other on-line methods e.g., using the fluorescence resonance energy transfer (FRET) principle could also be used.

2. Material and methods

For the double competitive PCR approach the RRS specific detection was performed essentially as described by Wurz and Willmund (1997). An internal standard consisting in a synthetic amplicon with identical Primer binding sites was reamplified and cloned yielding an amplicon with the following sequence:

TGTATCCCTTGAGCCATGTTGTTAAAGGGTCT
TGCGAAGGATAGTGGGAGCGGCCGCTGGATC
CGAATTCAAGCTCTTGCCTGGCATACGTCAGT
GGAGATATCACATCAATCAGCGCAATGATGG
CATTTGTAGGTG.

For the lectin le1 specific detection (see also Wurz et al., 1998) primers were: Forward (5′-GACGC-TATTGTGACCTCCTC-3′) and reverse (5′-TGTCAG GGGCATAGAAGGTG-3′). The standard amplicon was cloned after introducing a deletion using a linker primer, yielding the following amplicon:

GACGCTATTGTGACCTCCTCGGGAAAGTTACA
ACTCAATAAGGTTGACGAAAACGGCACCCCA
AAACCCTCGTCTCTTGGTCGCGCCCTCTGGAT
CCGAATTCAAGCTTTTCAACTTCACCTTCTATG
CCCCTGACA.

All PCR's were run on a Perkin–Elmer GeneAmp 9700 or 2400 in 25 μl reactions with 1U of Amplitaq Gold polymerase for 10 min/95°C followed by 50 cycles of [25 s/94°C, 30 s/62°C, 45 s/72°C] and a final 7 min/ 72°C.

For the TaqMan approach the same amplicons were used with exception of the reverse lectin le1 primer being 5′-TGTCAGGGGCATAGAAGGTG-3′ to ensure amplicons for both systems being approximately of the same size. This is to avoid bias caused by highly fragmented DNA as template (as may occur in processed foods). Internal probes labelled 5′-FAM and 3′TAMRA were used for detection during a standard two step PCR-profile of 10 min/95°C followed by 50 cycles of [15s./95°C, 1min/60°C] on an AbiPrism 7700.

Cloning and post-PCR steps were performed according to standard procedures. DNA samples from soya reference material (Fluka 85,473) were prepared using a WIZARD™ or CTAB based methods.

3. Results

3.1. Double competitive PCR

At present no reference material for mixed food samples is available but, as is obvious, non-soy derived DNA will not interfere with the method presented in this chapter.

DNA was extracted from Fluka 0.5% and 2% GMO soya reference material. Using competitive PCR on the lectin le1 gene, the lectin gene copy number was estimated in a first step for both samples (data not shown, for pure products OD 260 measurement can also be used).

DNA extracts were then diluted accordingly, in order to match a lectin competitor-concentration matching 25,000 copies of the lectin amplicon per reaction. A second PCR specific for RRS was carried out, using the same amount of sample DNA and an amount of competitor matching 250 copies of the RR specific amplicon (for convenience the two competitors are used as premixed solution in defined ratios, yielding equivalent band intensities with defined copy numbers of the targets e.g., for 1% RR soy as in the example shown in Fig. 1). The PCR products were then separated and visualized on an agarose Gel by ethidiumbomide staining (Fig. 1).

By simply comparing the band intensities a sample can easily be evaluated with respect to the threshold that was predefined in the experiment.

Theoretically this technique may also allow the determination of exact values of the ratio of the two targets to be determined, but using such a simple non-radioactive approach and a competitor amplicon differing substantially in length would need substantial effort to do so. The generation of calibration curves is rather complex and the densitometric measurement suffers from general problems in connection with staining-properties, diffusion phenomena etc. Thus this technique will be rather suited for threshold determinations than for calculation of actual ratios.

3.2. On-line PCR measurement (Abi-prism 7700)

The so-called TaqMan method exploits the 5′–3′ exonuclease activity of some Taq-polymerases to

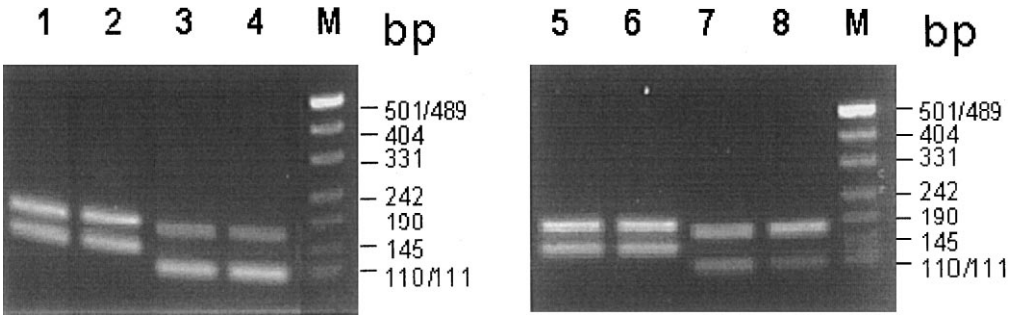


Fig. 1. It shows the amplification products after coamplification of the same volumes of DNA extracted from 0.5% (Lanes 1–4) and 2% (Lanes 5–8) Fluka reference material together with 25,000 copies of lectin le1 competitor using the lectin specific primers (Lanes 1–2 and 5–6) and 250 copies of the RRS specific competitor using the RRS specific Primers (Lanes 3–4 and 7–8) Two parallel PCR are shown for each sample. The upper bands represent the two original amplicons the lower ones represent the two (smaller) competitor amplicons. In Lanes 1–2 and 5–6 lectin le1 is "matched" as indicated by same band intesities for both amplicons in the reaction. In Lanes 3–4 the competitor band is stronger than the original RRS band thus indicating that the sample is below 1% RRS. In Lanes 7–8 the competitor signal is weaker, thus indicating the sample to be above 1% in RRS content.

generate a template-specific fluorescent signal after hydrolysing an internal probe during each step of the PCR. The resulting kinetics reflects the integration of this exponential process. The parameter measured is the threshold-cycle (CT) where each reaction trespasses a certain fluorescence level.

Using two standard curves for both PCR systems (RRS and lectin specific) the initial template concentration can be calculated and the ratio can be determined.

Using the above mentioned Fluka standard material for GMO soy, we evaluated the TaqMan method as outlined above: Results (see Table 1) for the GMO ratios are means of two independent determinations of the same DNA sample.

The 0.1% sample was determined as 0.07% – considering the fact that the underlying RRS-copy number was in the range of only 10 copies, statistical effects may contribute substantially to the error observed. For the values of 0.45% for the 0.5% standard and 1.8% and 1.91% for the 2% standard relative errors are less and will reflect rather system inherent than statistical effects.

3.3. Discussion/conclusions

Our aim was to develop methods for the quantification of GMO in mixed and processed foods.

The competitive PCR approach does not need special equipment and is therefore suited for transfer to a broad spectrum of laboratories involved in GMO testing by PCR. It uses derivatives of already standardised and well-known detection systems.

Another important aspect of using internal standards is the fact of improved control of false negative results caused by inhibition effects. Whereas simple qualitative PCR might suffer from false negative results (e.g., if the positive control is another PCR system or uses higher copy numbers) the use of an internal standard, close to the detection limit (e.g., 10 copies/reaction), gives reliable control of PCR performance, thus reducing inter-laboratory differences with PCR sensitivity.

Inhibition effects will most probably have no substantial influence on the quantification as long as a reasonable signal can still be obtained. This is due to the fact, that amplification reactions for both targets share all components except the target itself.

During development of internal standards the virtual identity of amplification efficiencies for both targets is an important prerequisite that must be considered (data not shown). Otherwise the system becomes prone to bias by inhibitory effects and absolute target concentration in the PCR, as in these cases the cycle number for entering the PCR plateau would vary for given target concentrations. Therefore criteria should be developed for the establishment of such systems.

In the example shown, a 0.5% and a 2% RR-content could clearly be distinguished. However some extent of uncertainty for samples ranging close to the threshold that was set, cannot be avoided. Thus the exactness of the method in routine application should be improved and addressed e.g., in inter-laboratory studies.

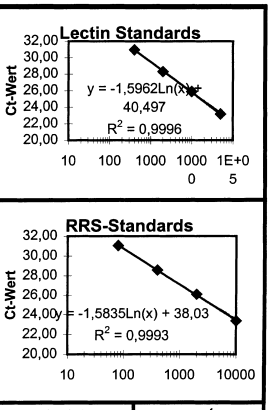
The second approach using the on-line measurement of PCR offers three main advantages:

- It allows individual ratios of GMO content to be calculated and is well suited for automation and high throughput of samples.
- Furthermore the signals detected are sequence specific, thus giving additional evidence for the identity of the amplified targets.
- It can also be used for raw, processed or even mixed products.

Whether the exactness of the method can still be increased needs further research. It must be considered that error development is quadratic when calculating ratios and that PCR is still being an exponential process. Especially for its application in processed products a system with reduced amplicon sizes is currently

Table 1
The table shows the results of the run of 0%, 0.1%, 0.5% and 2% Fluka reference samples on the Abi-Prism 7700[a]

Sample		Quantity	Ct	Mean of two replicates	copy number	percentage RRS
Lektin-St.	Replik 1	50000	23,09	23,19		
	Replik 2	50000	23,29			
Lektin-St.	Replik 1	10000	26,03	25,89		
	Replik 2	10000	25,74			
Lektin-St.	Replik 1	2000	28,23	28,30		
	Replik 2	2000	28,36			
Lektin-St.	Replik 1	400	30,94	30,95		
	Replik 2	400	30,96			
RRS-St.	Replik 1	10000	23,33	23,37		
	Replik 2	10000	23,4			
RRS-St.	Replik 1	2000	26,04	26,11		
	Replik 2	2000	26,17			
RRS-St.	Replik 1	400	28,65	28,57		
	Replik 2	400	28,48			
RRS-St.	Replik 1	80	31,11	31,04		
	Replik 2	80	30,97			
No Template	Lektin 1		50	50,00	0	(No Template control)
	Lektin 2		50			
	RRS 1		50	50,00	0	
	RRS 2		50			
Fluka 0%	Lektin 1		26,47	26,43	6720	0,00%
	Lektin 2		26,39			
	RRS 1		50	50,00	0	
	RRS 2		50			
Fluka 0.1%	Lektin 1		24,83	24,93	17252	0,07%
	Lektin 2		25,02			
	RRS 1		33,81	34,15	12	
	RRS 2		34,49			
Fluka 0.5%	Lektin 1		25,71	25,69	10716	0,45%
	Lektin 2		25,66			
	RRS 1		32,22	31,89	48	
	RRS 2		31,56			
Fluka 2%	Lektin 1		25,32	25,39	12892	1,80%
	Lektin 2		25,46			
	RRS 1		29,5	29,41	232	
	RRS 2		29,31			
Fluka 2% diluted 1:2	Lektin 1		26,59	26,39	6890	1,91%
	Lektin 2		26,19			
	RRS 1		30,33	30,30	132	
	RRS 2		30,27			
Simulation 1% Level	Lektin 1	undiluted	23,12	23,06	55496	0,93%
	Lektin 2	100% RR	23			
	RRS 1	1:100 dil.	28,13	28,15	514	
	RRS 2	100% RR	28,16			



Lec. slope:	-1,5962	RRS slope:	-1,5835
Lec. Y-interc.:	40	RRS interc.:	38,03

[a] All determinations including the standard curve samples were done in parallel and the average of two experiments was calculated. Means of the Ct values of the samples together with slopes and Y-intercepts of the two standard curves shown, lead to the calculation of the copy numbers indicated in the sixth column. Ratios were calculated using these averaged copy numbers (column 7). The last four rows show a simulation experiment in which the DNA (100% RRS) was used undiluted for lectin le1 determination and was diluted 100 fold for the RRS determination, to simulate the 1% level.

developed in order to extend the range of products that can be analysed.

Within the scope of this contribution no quantified reference material for mixed matrices was available, but as both methods use species specific reference templates, other DNA is not supposed to interfere with the measurements.

Finally the issue of efficient DNA extraction methods from different types of processed foods that are suitable for routine analyses has not been addressed in the above experiments. Wether or not a food can be analysed by the quantitative means described above is highly dependent on the availability of such extraction methods. It can be calculated, that for reliable quantification at the 1% level, at least 10,000 copies (amplifiable DNA) of the soy genome should be present in the PCR reaction – this is estimated to be $\sim$ 50 ng. On the other hand purity of the DNA limits the amount to

be put into PCR assay with respect to inhibitory effects.

3.4. Future prospects

Quantitative PCR-based methods can be extended to any given GMO – even a quantitative screening e.g., using the 35S promotor as a target has successfully been done. Future activities should focus on the development of more transgene – and also species specific PCR systems (a maize system has already been developed in our lab).

First attempts to standardize quantitative methods have been made but they clearly must be reinforced. Providing pure and mixed reference material as well as sequence information of approved GMO is a problem, that should be addressed by the legal authorities during the process of approval of novel food products, as otherwise proper labelling cannot be ensured.

Furthermore the development of routine methods for efficient extraction of well amplifyable DNA constitutes a main challenge, when considering the heterogenity of food matrices and raw materials to be analysed in the future.

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