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Suitable method for simultaneous and specific detection of maize (*Zea mays* l.) and genetically modified soyabean (*Glycine max* l.) in animal feeds*

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ABSTRACT

Present method allows a simple and rapid identification of maize and specific transgenic soyabean event in raw materials and processed feed samples. The method combines two aspects: suitable DNA purification and amplification by means of multiplex RT-PCR. Efficiency and accuracy of this method have been tested and the limit of detection (LOD) reached was of 0.1 ng/µl for one of the species and of 1 ng/µl for the other one. The checking of the method has been realized by means of RT-PCR using the specific probes for each one of the systems by means of comparison with certified reference materials. The obtained results showed that with this method it is possible to obtain, in a shorter time, quality values for both: identification and quantification of GMOs, in raw and processed samples.

KEY WORDS: feed, maize, soyabean, GMO, transgenic, RT-PCR

INTRODUCTION

The use of high-quality raw material in feed production is considered to be a prerequisite factor to obtain a genuine and secure product of adequate nutritional value. The European Union imports large amounts of feed from third countries in which GM (genetically modified) crops are cultivated. There are potentially import market impacts in the EU linked to possible shortages in supply and

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the need for switching to alternative products of inferior quality and/or higher prices (European Commission, 2007). Furthermore, traders would be unwilling to assume the risk of having traces of EU nonauthorized genetically modified organisms (GMOs) detected in their shipments. For these reasons, in the European Community traceability of the origin, quality, and authenticity of feed products is becoming very important. Consequently, it will become necessary to develop appropriate techniques to trace and label feeds correctly. There is a need to develop reliable detection methods (Peano et al., 2004).

The methods of molecular analyses, as the ELISA test, PCR (Polymerase Chain Reaction) and RT-PCR (real time-PCR) have turned out to be very efficient and economically profitable in the development and follow-up of the varietal identification and genetically modified organisms (Germini et al., 2004; Gómez and Alonso, 2004; Fortea et al., 2005; Gómez et al., 2007). The results obtained from these analyses are being used to take critical decisions to all the levels from the discovery of the genes, the varietal identification, the genetic transformation process and the selection, up to the quality controls, the traceability and the labelling. Although the same technologies could be used from the analysis of seeds or another plant material to the processed materials, the sampling strategies, the protocols for DNA extraction, the reference standards and the interpretation of the results must be adapted to every purpose (Grothaus, 2000). Specially, sample matrix and DNA extraction protocols have been shown to affect PCR kinetics (Cankar et al., 2006).

Among all these molecular technologies the PCR, and moreover real-time PCR (RT-PCR) has acquired a great value, allowing the detection of genetically modified organisms, the crossings between them and non-modified organisms (Tang et al., 2004) and their presence in mixed samples. The simultaneous determination of the presence of different genes of interest in the samples can be realized by means of the technology named multiplex-PCR. Multiplex-PCR improves the process since it manages to amplify simultaneously and in a single reaction tube different DNA fragments.

The simultaneous amplification needs a suitable design of the reaction. It implies the choose or the design of primers that do not interact between them, that have similar annealing temperatures, every pair must amplify a specific fragment, and generated amplification fragments must have enough different sizes to allow their separation and clear identification after the amplification process.

We describe here a sensitive and specific method to rapidly and simultaneously identify the most common genetically modified plants used in feed compositions by multiplex-PCRs. The method combines species-specific primers and event-specific primers in a single PCR tube. To obtain genomic DNA template, we demonstrate that small quantities of feeds can be sampled and tested directly with an easy and rapid protocol.

MATERIAL AND METHODS

Samples

Reference materials of soyabean Round-up Ready™ and maize MON-810™ have been obtained from the Institute for Reference Materials and Measurements (IRMM-JRC, European Commission). The analysed samples were five raw materials, twenty two processed feeds and fourteen complex synthetic maize-soyabean samples with DNA in different proportions (Table 1).

Table 1. Raw materials, processed feeds (P) and complex synthetic maize-soyabean samples (S) used in this study.

Synthetic samples			Feed samples		
code	soyabean proportion	maize proportion	code	% GM soyabean	presence of maize
S1	1	9	P1-raw	≥5%	-
S2	2	8	P2-raw	≥5%	-
S3	3	7	P3-raw	-	+
S4	4	6	P4-raw	-	+
S5	5	5	P5-raw	No detected	-
S6	6	4	P6	≥5%	+
S7	7	3	P7	≥5%	+
S8	8	2	P8	2.8%	+
S9	9	1	P9	≥5%	+
S10	95	5	P10	≥5%	+
S11	96	4	P11	≥5%	+
S12	97	3	P12	≥5%	+
S13	98	2	P13	≥5%	+
S14	99	1	P14	≥5%	+
			P15	≥5%	+
			P16	≥5%	+
			P17	≥5%	+
			P18	≥5%	+
			P19	≥5%	+
			P20	≥5%	+
			P21	≥5%	+
			P22	≥5%	+
			P23	≥5%	+
			P24	≥5%	+
			P25	≥5%	+
			P26	≥5%	+
			P27	≥5%	+

DNA extraction methods

Feeds were prepared by grinding 10 g per sample at 25 vib/sec for 30 sec in a mixer mill (MM 301, Retsch).

CTAB method. DNA was extracted from an amount of 100 mg from each milled feed or reference material flours by means of a CTAB-DNA extraction and purification protocol. The protocol has been validated for soyabeans (Anon, 1998) and for maize by JRC (Joint Research Centre, European Commission). The protocol is an enhanced CTAB-DNA extraction and purification protocol adopted from the prEN (ISO 21571:2002).

Commercial Kit method. DNA was extracted from an amount of 70 ng from each milled feed or reference material flours by using PrepMan® Ultra reagent (Applied Biosystems, Rotkreuz, Switzerland) according to the instructions of the manufacturer. The quality of the extracted DNA was monitored with a UV/Visible spectrophotometer (BioPhotometer, Eppendorf, Germany) and the concentration of total DNA was determined with the procedure from the Anex B "Methods for the quantification of the extracted DNA" of the prEN ISO 21751:2002. Each measurement was repeated twice.

PCR amplifications

The amplifications were carried out in 20 µl volume reactions, with 48 ng sample DNA, 1x PCR buffer, 200 µM dUTP, 4 mM $MgCl_2$, 0.75 µM primer pair, 0.2U AmpErase™ uracil N-glycosylase (Applied Biosystems, California, USA), and 1U AmpliTaq Gold polymerase (Applied Biosystems, California, USA). The reaction was performed in the Applied Biosystems, 2720 Thermal Cycler with 10 min at 95ºC, followed by 50 cycles of 15 s at 95ºC and 1 min at 60ºC.

For detection and relative quantification of event Round-up Ready™ DNA, a soyabean-specific reference system amplifies a 118-bp fragment of lectin, a soyabean endogenous gene, using a pair of lec primers and Lec probe labelled with FAM and TAMRA. For Round-up Ready™ a pair of gene-specific rr primers and rr gene-specific probe were used (Kuribara et al., 2002). The detection of the intrinsic gene *adh1*, a maize endogenous gene, was performed by amplifying a 70-bp fragment of *adh1* (JRC, 2005).

The amplification products were visualized through electrophoresis on 2.5% agarose gels after ethidium bromide staining. The expected size of the amplified fragments was estimated by comparison with Biomarker Low (Bioventures Inc., Murfreesboro, USA).

Soyabean flour with a content of 5% genetically modified soyabean (IRMM-410) and maize (IRMM-413) have been used for the construction of the standards curves used in the quantification by means of RT-PCR. RT-PCRs were performed

in an ABI 7500 System (Applied Biosystems, California, USA). Results have been analysed as shown in Table 2.

Table 2. Nomenclature used for RT-PCR and conventional PCR results

RT-PCR Result	PCR result	
	positive	negative
Positive	III	II false negatives
Negative	I false positives	IV

RESULTS AND DISCUSSION

DNA quantity and quality. The DNA samples extracted from feeds containing soyabean and/or maize materials, with the two extraction methods, were evaluated. As long as, PCR methods can be successfully applied for the feed analysis, thanks to their high specificity and sensitivity as well as to their rapidity, they are mainly limited by the presence of inhibitors derived from DNA extraction (Wilson, 1997). Moreover, DNA degradation due to DNA extraction protocol or feed processing may degrade DNA molecules and introduce substances that interfere with the PCR, leading to ambiguous or false results. The DNA extraction, purification and concentration, the first critical step in molecular analytical methodologies, requires methods able to remove the several inhibitor compounds of the amplification reaction. (Di Pinto et al., 2007).

By a literature search, the most commonly used DNA extraction method for the detection of genetically modified organisms in food was selected, this is the CTAB method validated by the JRC (Join Research Centre, European Commission). The other purification system used is the PrepMan® Ultra reagent (Applied Biosystems) which is applicable for a variety of different sample preparation applications (see manufacturer indications). It has been used successfully to prepare DNA template from bacteria (Rudi et al., 2004; Tannaes et al., 2005), from fungus (Morgan et al., 2007), from animals (Babaglio et al., 2006) and foods (Vodret et al., 2007).

To simplify determination of how much DNA was recovered the input weight for each DNA extraction method was the same for all the samples and the output volume (100 µl) was fixed. After DNA extractions the concentration of total DNA was determined. Table 3 shows mean DNA output (ng/µl) with

Table 3. Averaged extracted DNA from raw materials and processed feeds by CTAB and PrepMan® Ultra DNA extraction methods

Sample	DNA, ng/µl	
	CTAB method	PrepMan® Ultra
Raw materials	391.2 ± 40	402.5 ± 55
Processed feeds	724.2 ± 48	408.2 ± 43

two replicates per sample. The quantity and quality of the extracted DNA from samples that differed in their degree of processing and in the DNA extraction protocol used (CTAB buffer or the commercial kit) were compared. All samples yielded significant amounts of DNA. DNA yields from raw materials varied from 138 to 610 ng/µl (138 to 610 ng DNA mg^{-1} flour) with CTAB and from 80 to 690 ng/µl (114 to 986 ng DNA mg^{-1} flour) using the commercial kit. DNA yields from processed feeds varied from 198 to 1330 ng/µl (198 to 1330 ng DNA mg^{-1} flour) with the CTAB method and from 122 to 735 ng/µl (174 to 1050 ng DNA mg^{-1} flour) using the commercial kit. In general processed feeds yielded higher quantities of DNA than raw material samples with CTAB protocol. The high standard deviations obtained mean that there are great differences in DNA quantity obtained between samples. In this sense soyabean raw materials showed highest differences in quantities between samples than maize raw materials. It could be related with DNA purity.

DNA concentration and purity, estimated by measuring the A260 absorbance and A260/280 absorbance ratio respectively, were discordant. In particular the spectrophotometer analysis on DNA purified by CTAB protocol. The A260/280 absorbance ratio was comprised between 1.70 and 1.77. On the other hand, similar A260/280 absorbance ratios (1.68 and 2) were obtained for samples extracted by means of PrepMan® kit. Pure dsDNA ratio (A260/A280) should be 1.8, presence of proteins can decrease the ratio value while the presence of RNA in the extract could be responsible for the ratio near 2 (Wilfinger et al., 1997).

Single marker PCR assays. To verify DNA suitability for PCR species-specific and event-specific PCRs were performed. Furthermore, RT-PCR was performed in order to verify the authenticity of the bands detected by conventional PCR and agarose gels electrophoresis, thanks to the use of the specific probe. The PCR assays highlighted that DNA extracts from raw materials and processed feeds, by both extraction methods, were suitable for PCR amplification. For the species-specific markers results were independent from the DNA extraction followed protocol (Table 4). In the case of event- specific markers differences were found between the extraction methods (Table 5). No Type II errors were detected, that means no false negative results were obtained when conventional PCR was

Table 4. Raw materials and feed samples duplicated analysis of *lec* fragment (soyabean specific fragment) and *adh1* fragment (maize specific fragment)

RT-PCR result	PCR result *lec*		PCR result *adh1*	
	positive	negative	positive	negative
Positive	50	0	48	0
Negative	0	4	0	6

Table 5. Raw materials and feed samples duplicated analysis of *rr* fragment (soyabean MON-Ø4Ø32-6 specific fragment)

RT-PCR results	PCR results *rr*			
	CTAB		KIT	
	positive	negative	positive	negative
Positive	38	0	38	0
Negative	7	9	0	16

used for both, CTAB and PrepMan®-Kit. By the other way, Type I errors were detected, these false positives results were obtained when conventional PCR and CTAB method were combined for the soyabean roundup ready specific marker. The most common cause of false-positive results is contamination with previously amplified DNA. Normal practices in the Biosafety Lab. include the use of separate areas for sample preparation, PCR and electrophoresis and the use of Amperase-UNG and dUTPS in order to avoid the presence of preamplificated fragments as templates. In this sense, the UNG does not have any significant effect on the PCR efficiency (Pennings et al., 2001). Furthermore the use of real-time RT-PCR helps mitigate this problem by operating as a contained system.

The explanation must be the amplification of a non specific fragment, nonspecific amplification is mainly caused by nonspecific primers. Really, BLAST analysis (Altschul et al., 1997) of this *rr* product's sequence shows that it is amplified from another *Glycine max* L. DNA fragment (Accessions: AC186737 and AC170861).

Although DNA extraction is a critical point for application of this technique, looking at our results we can conclude that both methods are appropriate for extraction of DNA from feed. The CTAB method used in a lot of laboratories, needs more time for its execution while the PrepMan®-kit has the advantage of being fast, avoiding false positives in conventional PCR, and furthermore, the quantitative results obtained are comparable to those from the CTAB technique.

Multiplex PCR assays. The multiplex-PCR limits of detection (LOD) were considered to evaluate the performances of the assay. In this analysis for specific PCR genes, LODs were established by preparing complex mixes of soyabean-maize DNAs (samples S1 to S14) and three replicate measurements were performed (an example is reported in Figure 1). The analysis showed the lack of blank signals (L10 and L11; Figure 1) were none of the templates, except those for each species-specific fragment, elicited any amplification, confirming the specificity of the assay. Therefore we set the trustable thresholds which were significantly different from the background values and resulted in LOD values of 0.1 ng DNA/µl (*adh1*) and 1 ng DNA/µl (*Lec*).

For RT-PCR amplifications of both specific fragments, first, the method calibration curves were designed with each primer/probe system using a 1:1

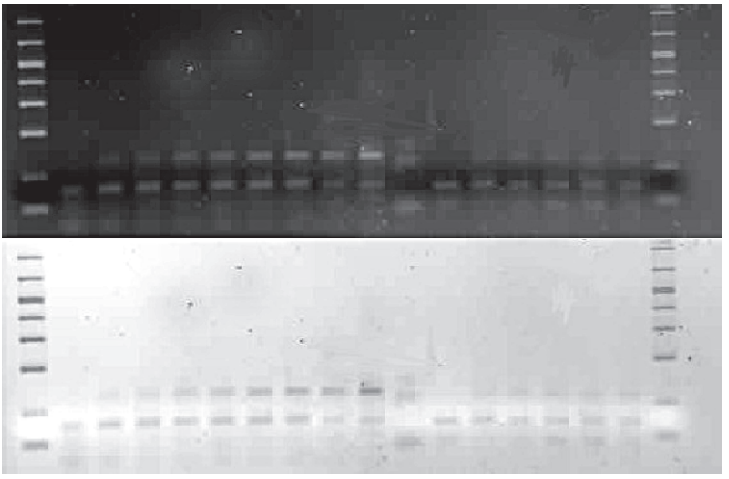

Figure 1. Agarose gel. PCR amplifications of *adh1* and *Lec* fragments from complex mixes (soyabean:maize). Lines M: MW marker; L1 (Line 1) - S9; L2 - S8; L3 - S7, L4 - S6; L5 - S5; L6 - S4; L7 - S3; L8 - S2; L9 - S1, L10 - control + lec; L11 - control + *adh1*; L12 - S10; L13 - S11; L14 - S12; L15 - S13 y L16 - S14

(soyabean:maize) DNA mix. The calibration curves consist of five samples. The first sample (M1) was a 1:1 (soyabean:maize) DNA mix for a total of 200 ng of DNA. It corresponds to 36.697 maize genome copies with one genome assumed to correlate to 2.725 pg of haploid maize genomic DNA (Arumuganathan and Earle, 1991). And, in addition, to 13.467 soyabean genome copies because the haploid genome size of soya is approximately 1.25 pg (Genome size taken from the Plant DNA C-values Database, Royal Botanic Gardens, Kew, UK). The other samples comprise a series of one to three dilution intervals (one to four to the last two dilutions) down to 1.4 ng of total DNA-mix/sample (M5) starting from M1. These analyses were performed three times in order to verify the results. The quantification by multiplex RT-PCR of the content of the samples in *Lec* and *adh1* (Table 6) showed a high correlation (r^2= 0.99 for *Lec* and 0.97 for *adh1*). The quality of multiplex PCR was confirmed by a statistical analysis based on Student's *t* test for coupled samples (quantities of each specific fragment determined by realtime-PCR and real quantities). No significant differences were found.

Table 6. *t* test for coupled (M1 to M5) samples and real quantities at different DNA levels with the both analysed fragments (*Lec* and *adh1*) through multiplex-PCR

Fragment	*t*	*P(t)*
Lec	1.87	0.135 ns
adh1	1.36	0.245 ns

t - t coefficient; *P(t)* - significance of t; ns - P>0.05

Then, multiplex RT-PCR was performed for samples M1 to M9 using the previous separate calibration curves with each primer/probe system, now generated in the same analytical amplification run. The copy numbers measured for the unknown sample DNAs were obtained by interpolation from the standard curves. The performance requirements were established at 0.99 R^2 coefficients, -3.4 and -3.1 slopes for *Lec* and *adh1* systems respectively. No significant deviations were obtained from the known DNA copies from both species in each sample. In order to check the method, feed samples which contain both, soyabean and maize, were analysed and in all cases both species were correctly detected in the samples.

Quantification of Round-up ready soyabean by multiplex RT-PCR was performed. In European Union Round-up ready soyabean is the only one authorized genetically modified event for soyabean and, nevertheless, there are diverse authorized events for maize which require each one their specific analysis.

Four samples were analysed: two feed samples, P8 and P9 due to their different *rr*-soyabean content previously quantified by conventional RT-PCR (2.8% and >5%) and the other two derived from S7 (S7 mix contains, approximately, the same genome copies from maize and soyabean) and 5% presence of *rr*-soyabean in total soyabean. S7-1 means 2.66 ng per µl of reaction and S7-2 means 0.056 ng per µl of reaction. The performance requirements were established at $R^2 = 0.99$ and slope=-3.5 for *rr*-system. The quality of this multiplex RT-PCR was confirmed by a statistical analysis based on Student's *t* test for coupled quantities of round up-ready soyabean determined by multiplex RT-PCR and conventional RT-PCR (Table 7). The data showed a high correlation $r^2=0.97$ and no significant differences were found.

Table 7. *t* test for coupled samples and real rr-soyabean quantities and at different DNA levels with the both analysed fragments (*Lec* and *rr*) through multiplex-PCR

	t	*P(t)*
rr-soyabean, %	-0.827	0.469 ns

t - t coefficient; *P(t)* - significance of t; ns - P>0.05

The accuracy of the method, it means the closeness of agreement between a test result and the accepted reference value, was evaluated for the proposed multiplex RT-PCR method. For all the tested samples and systems (*Lec, adh1* and *rr*%) the accuracy of the method was comprised within ±25% of the accepted reference value.

Our data show that the commercial kit and the multiplex RT-PCR have the advantage of being fast and reliable for simple and processed feed. The real time PCR technique is widely known as the most sensitive and highly specific method

for GMO quantification. Nevertheless, processed feed suffer treatments like grinding and heated up at high temperatures which can distortion the quantification of GMO in the samples (Moreano et al., 2005). In this sense, we have compared both methods, conventional and proposed multiplex RT-PCR, and comparable results were obtained without significant differences for raw and processed feed.

CONCLUSIONS

In the European Community traceability of the origin, quality, and authenticity of feed products is becoming very important. In this sense the laboratory analyses are being used to take critical decisions to all these levels. With this purpose, present method (PrepMan® ultra followed by multiplex RT-PCR) can be used to obtain accurate answers in a short time in GMO monitoring in feed, with the aim of performing screening and/or quantification analysis of different crop species by real time PCR.

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