Reliability of PCR based Screening for Identification and Quantification of GMOs

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Abstract

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Handling with genetically modified organisms (GMOs) is regulated namely in EC. Laboratories often use polymerase chain reaction (PCR) based screening methods to monitor the presence of GM particles in food commodities as a cost effective approach. The reliability was tested of such screening using 35S CaMV promoter as the target sequences. Soya grown from non-GM cultivar as declared by a seed company was investigated after the harvest, transport to the silo, and before processing. The results based on PCR and real-time PCR analysis clearly showed that, the contamination with debris of other species, dust during transport, storage, and other kind of handling led to contamination with detectable amounts of *Cauliflower mosaic virus* (CaMV). Impurities are allowed by EC regulations but may, as we have shown, interfere with the analytical procedures based on PCR. The identification of 35S CaMV promoter and NOS terminator in food with uncertain history and no approved specific events may indicate unknown GMOs and perhaps emergency situation.

Keywords: GMO (Genetically Modified Organism); identification; PCR; real-time PCR; screening; RR soya

Genetically modified plants (GMPs), that means plants whose genome was modified by insertion of one or more genes into the genome in a way that does not occur naturally, have become a part of the food chain worldwide and GMPs production has been constantly increasing (James 2008). Compared with other parts of the world, EC has approved only a limited number of GM events, most of which are imported anyway. On the whole, European food producers reflect the sensibilities of their consumers and prefer to use GM-free ingredients, thus primarily corresponding cultivars are used on EC farms. It is only corn MON810 which is cropped to some extent (Anonymous 2008). Although the approved GM events must be labelled on the food products containing them, in EC a tolerance of up to 0.9% exists for accidental and technically unavoidable admixtures (Commission Regulation (EC) 1829/2003). GMO handling is controlled by competent authorities in each country, and incorrect handling can lead to administrative affairs and penalties. For this reason, food producers run their own internal audits and, more commonly, submit either properly

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sampled materials or randomly selected samples for laboratory analysis.

PCR (Polymerase Chain Reaction)-based methods have been developed and validated for the detection of GMOs and derived products in the food chain and PCR constitutes the most common approach (Garcia-Canas *et al.* 2004; Michelini *et al.* 2008). PCR, which enables multiple DNA sequences to be targeted by unique primer sets, is used not only to confirm the presence of a particular DNA sequence, but also to quantify it (Elenis *et al.* 2008; Marmiroli *et al.* 2008). Even several copies of target transgenes may be detected and quantified by the approved methods. Their sensitivity is given as Limit of Detection (LOD) or Limit of Quantification (LOQ), respectively. In EC such detection and quantification methods are normally validated in ring trials organised by the Community Reference Laboratory (CRL) at the Joint Research Centre in Ispra, Italy, which was established in accordance with EC regulations. The CRL is assisted by the ENGL (European Network of GMO Laboratories), which carries out validation studies in accordance with EC regulation 1829/2003. Only methods meeting the specified performance criteria are approved as suitable for the purpose (Zel *et al.* 2008). The methods and their updates are available on the CRL website (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

Most EC-approved GMPs were developed using the 35S CaMV (*Cauliflower Mosaic Virus*) promoter and/or the NOS terminator derived from *Agrobacterium tumefaciens*. These elements are present in the first generation of GM material, and they are used for GMO screening (Vollenhofer *et al.* 1999) and in some cases also for transgene quantification (e.g. TaqMan GMO 35S Soya Detection Kit, Applied Biosystems; TaqMan GMO 35S Maize Detection Kit, Applied Biosystems). As the analysis of a specific GM event often requires multiple PCR reactions, it is expensive and time consuming. Consequently, the screening methods that only detect regulatory elements are considered more cost effective (Vollenhofer *et al.* 1999) when multiple samples have to be processed.

We investigated the ability of the screening methods to identify reliably the presence of GMO. We applied the method throughout the transportation/production chain from the farm to the factory. We chose soya as a model because soya is the most cultivated GM plant in the world, with 75% of the world soya production resulting from this cultivar (Anonymous 2008). GM Roundup Ready transgene consists of 35S CaMV promoter, transit peptide, EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) tolerant to glyphosate, and NOS terminator. Although (EC) food producers generally aim to avoid GM products, the results of the screening tests show that their products are often contaminated nevertheless. The results based on such screening occasionally appear in the European rapid alert system. It was this fact that prompted our investigation.

MATERIALS AND METHODS

Plant material. All samples of soya kernels originated from a field in the Czech Republic in which a non-GM cultivar was grown as declared by the seed company. The samples were taken at three sampling points: the first sample was taken directly after harvesting, prior to the kernels being loaded into trucks, the second was taken after transportation, during offloading from the trucks into a silo; and the third was taken prior to the processing of the grains in the factory. At each sampling point, triplicate samples were taken. For sampling from the end-gate grain stream, increment samples from the entire width and depth of the grain stream were collected. Double-tube compartmented probes were used to collect the increment samples from lots of grain in carloads (stationary sampling). The grains from the first parallel were ground as they were harvested. Prior to analysis, visible impurities were manually removed from the grains in the second parallel, while grains in the third parallel were manually purified, carefully washed with sterile distilled water and dried.

DNA isolation. The soya kernels were ground into a homogenous powder with an average particle size of 200 μm. The DNAs were extracted and purified in accordance with the procedure prescribed in EN ISO 21571:2002. Two independent isolations were performed from each analytical sample. DNA quality and quantity were then estimated following the procedure described in Appendix B of the same standard.

GMO detection. The ability of DNA amplification was verified using primers specific for the lectin gene. As described by Vollenhofer *et al*. (1999), PCR was used to detect the presence of GM elements for the 35S CaMV promoter, NOS

terminator, and EPSP synthase. The CaMV sequence itself was detected following Wolf *et al.* (2000), using 100 ng of the extracted total genomic DNA per reaction. The detection limit of the reactions was estimated as 30 copies of the target sequence with an expected confidentiality of 98% in soya flour using the equipment and personnel available in the laboratory. DNA extracted in the same way from IRMM RR soya standards ERM BF410 (Sigma-Aldrich, St. Louis, USA) were used as a control.

GMO quantification. GMO quantification was performed using the TaqMan® GMO 35S Soya Detection Kit on ABI 7900 HT (both Applied Biosystems, Foster City, USA). To verify the results, quantification was done using TaqMan probes specific for EPSPS (Pietsch & Waiblinger 2000) with the exception of TaqMan probe labelling – VIC and FAM dyes as well. GMO content was assessed relatively using Ct of each reaction for endogene and transgene and the calibration curve developed from IRMM standards.

RESULTS AND DISCUSSION

We investigated how reliably the screening PCR-based methods may detect the accidental presence of GM material in soya, as the screening methods are most commonly used (Morisset *et al.* 2008). With the increasing number of GMOs on the market equipped with more gene-specific regulative elements (Jacobsen & Schouten 2007), it is clear that the exclusive use of the 35S CaMV promoter and NOS terminator in screening will lead to false negative results in the future. However, we tested whether or not the sole use of these elements can also easily produce false positives. Roundup Ready (RR) soya is the most widely cultivated GM plant variety in the world (Akhond & Machray 2009) and is used as feed and food. Its transgene consisting among others of the 35S CaMV promoter derived from the cauliflower mosaic virus, a widely occurring pathogen of the *Brassicaceae* family (Haas *et al.* 2002); and the NOS terminator derived from the soil bacterium *Agrobacterium tumefaciens* (Yuan *et al.* 2008) were the analytical targets. The cauliflower mosaic virus itself is not infectious for soya, and thus should not be present in the kernel. Also *A. tumafaciens* is not expected to be part of the harvest. Therefore, a combination of two assays amplifying the 35S CaMV promoter and NOS terminator should be sufficient for detecting the presence of transgenic soya.

We analysed the soya samples taken at each stage of the transport chain from the farm to the factory. The plants were grown from the seeds of a traditional soya cultivar that had been tested negative for the presence of p35S CaMV and NOS terminator, as well as for the transgene itself (construct specific test) according to the certificate. The first samples analysed, i.e. those taken immediately after harvesting, were tested as negative, too (Table 1). However, the samples tested after the transportation in a truck, together with those tested after being stored in a silo, were both positive for the screening elements. The validated TaqMan GMO 35S Soya Detection Kit using also 35S CaMV sequence as the analytical target was used to quantify the level of contamination, which was found to exceed 0.9%. The analytical parameters of the assays were found to be within the expected limits (Fernandez *et al.* 2005), i.e. the slope of the calibration curve ranging from –3.1 to –3.6, Ct value for the endogen in the range 21–23, and uncertainty expressed as standard deviation was estimated to be 30% in all the samples analysed. As GM admixtures have a negative impact on the value of goods and their position in the market, we attempted to determine the cause of the con-

Table 1. p35S CaMV content (in %) in soya lots from field to processing, as detected using TaqMan GMO 35S Soya Detection Kit

	Total sample	Manually purified (%)	Manually purified and washed by water (%)
Field	0	0	0
Van	over 5%	1.4	0.35
Silo	over 5%	1.7	0.51
After purification	1.5%	1.1	0.1

tamination. Using the construct specific marker system, we tested the samples for the presence of the Roundup Ready transgene itself, but the results were negative using both PCR and real-time PCR. As no other transgenic soya cultivar was in use anywhere in the world at the time, we assume that the lot could be contaminated by naturally occurring DNA sequences in the respective virus or organisms.

However, such an assumption can not be made with regards to the resulting food products of unknown history. Such products being tested positive for the screened elements and negative for the approved events should/must be considered to contain unapproved GMO and, in accordance with the current legislation, directly withdrawn from the market (Commission Regulation (EC) 1829/2003).

With the replicates of the samples taken at three different sampling points (field, van, and silo), we were able to analyse the samples from the truck and silo after purification: the remaining samples were manually purified and the debris of unknown origin was removed; additionally, the last portions were manually purified as described above, and carefully washed with sterile distilled water and dried.

All samples were subsequentlly subjected to analysis. Table 1 shows that the contamination percentage as detected by the kit decreased after purification.

Based on the quantification of 35S CaVM sequence, the samples taken from the van and silo were reported to be GMO positive in quantity exceeding 5%. After the analysis of the manually purified samples, the detected contamination in the van and silo samples dropped to 1.4% and 1.7%, respectively; after manual purification with washing it fell to 0.35% and 0.51%, respectively. Thus, even after such cleaning when all the impurities are removed, the analysis based on PCR screening methods results in contamination percentage still exceeding the legal limit of 0.9% for labelling (Commission Regulation (EC) 1830/2003).

As the cleaning of the kernel decreased 35S CaMV promoter sequence quantity and contamination with other transgene was not probable, we attempted at detecting the sequence of the naturally occurring virus. PCR primers were used amplifying part of the virus genome outside 35S promoter. All the positive samples were analysed and found to be contaminated with the cauliflower mosaic virus itself, suggesting that the contamination is spread by the debris and dust of other plant species collected during the transportation and storage process (data not shown). In particular, silos are often used to store several species subsequently, thus they are difficult to remain free of the remnants of the previous species, and so admixtures are allowed to some extent. Such contamination is legal and is referred to as botanical impurities by EC legislation and may reach up to 2%. Normally, this does not have any negative impact on other properties of the lot. However, PCR is highly sensitive and, as other authors have also shown, good at detecting even the slightest amounts of specific DNA sequences (Holst-Jensen *et al.* 2003). DNA extracted from botanical impurities and their pathogens may interfere with the analytical procedure. Likewise, transgene specific assays using DNA originally occurring in other species as e.g. *Bacillus thuringiensis* (Bravo *et al*. 2007) as a target may lead under some circumstances to false conclusions as well.

The indication of the presence of unauthorised or unknown GMOs leads to consequent analytical and legislative steps that have to follow. Our findings clearly show that the methods targeting the screening elements including those suited for quantification have only informative value, however, such approaches are still used (Oraby *et al.* 2005; Reiting *et al.* 2007). If the individual validated event specific real-time PCR assays are used instead, the process of analysis slows down and the prices for the analysis of one sample increase. For that reason, laboratories call for simple cost effective assays as suggested e.g. by Hamels *et al*. (2009) or Chaouachi *et al.* (2008). However, they need to be approved by practice. We suggest that the combination of screening and event specific methods is a possible solution. New approaches are indeed highly required to ensure food safety in EC (Holst-Jensen 2008).

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