CO-EXTRA

GM and non-GM supply chains: their CO-EXistence and TRAceability

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RE Restricted to a group specified by the consortium (including the Commission Services)	
CO Confidential, only for members of the consortium (including the Commission Services)	

1 Summary

This deliverable is a description of the results of studies performed in connection with task T6.6 “Assess target analyte stability on the basis of selected parameters idenfied as potential sources of instability” of the Co-Extra project. The nature of the deliverable is described as a “public” intermediary report on the potential effect on quantification of GMO content by a set of possible sources of of analyte instability bias. The purpose of the report is partly to assist the project partners (e.g. in WP4, task T4.1) to critically review the basis for their activities, partly to assist the stakeholders in interpreting analytical results and implementing analytical procedures that may minimise the risk of bias of the resulting GMO quantity estimates.

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Arne Holst-Jensen
Ph.D., senior scientist
National Veterinary Institute, Oslo, Norway
Co-Extra project manager for partner 7

2 Background

The most common approach to detect, identify and quantify the GM content of agriculturally derived products is by application of various derivatives of the polymerase chain reaction (PCR) technology, e.g. real-time quantitative PCR (Holst-Jensen et al., 2003). PCR is sensitive to nucleotide substitutions in the annealing sequence of primers and probes, and substitutions or insertions/deletions in the primer or probe annealing sequence or (parts of) the entire amplicon target sequence will effectively invalidate a PCR method. Therefore, the long term stability of the target sequence over successive generations in seed production and fields is a requirement for PCR method reliability. The amplification efficiency during the first PCR cycles is largely unknown, but Peccoud and Jacob (1996 and 1999) and Nogva and Rudi (2004) concluded that differential amplification efficiency is common during these first cycles, and that this may have a significant effect on the final quantity estimates. The importance of amplification efficiency was also highlighted by Cankar et al. (2006). Holst-Jensen & Berdal (2004) introduced the term PCR forming unit (PFU) to discriminate between target sequence copies that amplify and fail to amplify in PCR, respectively. This term is relevant in the context of matrixes where DNA may be damaged as a result of processing or where presence of PCR inhibiting substances is a problem.

Many agriculturally derived products are subject to processing. Consequently, the DNA is also potentially affected by these processes. Furthermore, the DNA extraction process and handling of DNA in the laboratory in connection with analyses of agriculturally derived products are specific processes which intentionally affect the DNA. Generally, processing is expected to reduce the amount of DNA, either due to removal or degradation of some of the DNA in the starting material. However, since PCR based analytical methods measure PFU rather than target sequence copies, it may actually happen that the PFU number increases after a specific process. This can be explained if removal of PCR inhibitory substances from the DNA makes a previously non-amplifiable DNA sequence copy amplifiable.

DNA based GMO quantification is performed by determining the relative ratio of PFU of (a) GMO specific target sequence(s) $\boldsymbol{GM_{PFU}}$ and (a) species specific reference gene(s) $\boldsymbol{REF_{PFU}}$, see e.g. Holst-Jensen et al. (2003). If this ratio, usually expressed as a relative quantity determined as

$$GM\% = \frac{GM_{PFU}}{REF_{PFU}} \times 100$$

is affected by one or more of these processes, the result may bias the GMO quantity estimate. A random effect may be observed where PFU numbers are low, due to stochastic variation in the distribution of discrete units in space (Peccoud and Jacob, 1996, 1999, Berdal & Holst-Jensen, 2001; Holst-Jensen et al., 2003). A systematic bias may be observed where one sequence is more severely affected than another. Greater reduction of $\boldsymbol{GM_{PFU}}$ than of $\boldsymbol{REF_{PFU}}$ will result in underestimation of the GMO quantity, while greater reduction of $\boldsymbol{REF_{PFU}}$ than of $\boldsymbol{GM_{PFU}}$ will result in overestimation.

Traditionally, sequence specific instability bias has only been considered as a problem for long vs. short amplicons (Bauer et al., 2003), but recently a number of publications have described problems also with the short amplicons typically used in real-time PCR assays. These problems may be explained as, or are claimed to be caused by, sequence specific instability bias (Trapmann et al., 2002; Nogva and Rudi, 2004; Corbisier et al., 2005; Moreano et al., 2005; Yoshimura et al. 2005a; 2005b; Cankar et al., 2006; Engel et al., 2006).

Possible sources of instability bias listed in these publications include the size (Moreano et al., 2005; Yoshimura et al. 2005a; 2005b; Engel et al., 2006) and the specific sequence

composition of the targeted DNA sequence, but also structural configuration (e.g. folding of ribosomal RNA genes), upstream and downstream sequence motifs and function in the cell may affect the amplifiability or stability of the sequence (Nogva and Rudi, 2004). Some of the studies have focused on different processing, such as heat treatment, mechanical shearing in combination with wet- vs. dry-milling and extraction method (Trapmann et al., 2002; Corbisier et al., 2005; Moreano et al., 2005; Yoshimura et al., 2005a; 2005b; Cankar et al., 2006; Engel et al., 2006).

Deviations from expected quantities are commonly reported when measurements based on DNA are made on calibrant material where the assigned GM quantity is based on mass ratios, e.g. certified reference material from the Institute for Reference Materials and Measurements (IRMM), Geel, Belgium. This phenomenon is discussed in detail e.g. in Holst-Jensen et al. (2006). Furthermore, sampling uncertainty and inherent performance characteristics of reagents, equipment and staff are other factors generally recognized as major sources of variability between analytical results obtained on related samples within and between laboratories, e.g. when importers compare certificates from different laboratories, or in proficiency testing schemes (Hübner et al., 1999; Holst-Jensen & Berdal, 2004; Thompson et al., 2006). However, the expertise gradually agrees that additional factors may need to be considered and, particularly if bias patterns can be identified, these need to be pinpointed and quantified to improve the reliability of analytical quantity estimation.

The modular approach for method validation and application, proposed by Holst-Jensen & Berdal (2004) postulates that it may be possible to combine different modules in a flexible manner, e.g. that it may be possible for the analyst to chose between alternative DNA extraction methods and alternative reference specific PCR methods. If particular modules or processes, including extraction methods, introduce a sequence specific instability bias, then that may affect the degree to which the modular approach can be implemented, i.e. as to which modules it may be possible to combine under specified conditions. Since modularity is a very attractive solution to methods developers, 'validators' and users, it is very important that the issue of sequence specific instability bias is investigated thoroughly. Notably, however, the potential bias on GMO quantity estimation is not limited to modular method application. If a traditional (global) method is applied to GMO testing, it is equally likely to be affected by differential analyte stability. Moreover, it may be easier to identify the bias by application of the modular approach to method validation, because the source of the bias may be pinned down more accurately, e.g. to specific base compositions, amplicon sizes, structural conformations and links to specific processes.

Within this task, possible analyte instability in the living GMO is investigated by two of the partners; CSIC and ILVO, together with one partner from WP5 (NIB, partner 6). The collaboration focuses on the in-field nucleotide variability in inserted and endogenous sequences. The other two partners involved in T6.6 have chosen to focus on processing effects, i.e. post-harvest analyte instability. Both approaches may provide better insight into sources of bias in analyte ratios in products subject to testing, and may successively provide a basis for developing guidelines for taking bias into consideration when analytical results are being interpreted. The results may also lead to adjustments of current testing regimes, e.g. choice of analytical targets, etc.

Task T6.6 is to some extent related to another task T4.1 "*Analysis, development and testing of validation procedures and guidelines for modular validation approach*" in WP4. The main difference between these tasks is that T4.1 should focus on implementation of modularity, and how measurement uncertainty can be dealt with accordingly, while T6.6 should focus on how particular processes or other parameters may affect the quantitative results obtained when GM and target specific analyte quantities are compared.

Many of the experiments are designed on the basis of the modular approach, and the results or these experiments therefore also provide a better basis for reshaping and refining the modular approach for method validation and defining the range and limits of application of the modular approach in routine diagnostics.

Experiments performed in T6.6 are designed to provide a better basis for evaluating the possible impact of specific processes on specific matrixes with respect to how the analyte ratio, and consequently the estimated GM content is affected. Each partner participating in T6.6 has focused on a limited set of processes and matrixes, and the results obtained so far are summarized in the following.

The partners involved in T6.6 are:

- P7 (lead partner), National Veterinary Institute, Norway
- P21, CSIC, Spain
- P22, ILVO, Belgium
- P24, GeneScan, Germany

3 Specific contributions from the partners

3.1 NVI, partner 7 and task leader

The NVI is performing the work with a model system comprised of three non-GM maize lines and five maize single copy gene real-time PCR modules validated in collaborative trials (Holst-Jensen, 2005). This is an example of applying the modular approach proposed by Holst-Jensen & Berdal (2004) experimentally. This will allow us to perform the work without having to worry about material availability and potential GM contamination of processing equipment such as grinders and blenders. The real-time PCR methods will act as model systems substituting for GM specific PCR methods. We believe this is justifiable, since the methods target independent genetic loci. However, we appreciate the fact that it may be difficult to assess whether effects may result from target quantity differences like those found in e.g. a 1% GM material.

The single copy number status of the five methods has been determined independently by application of Southern blot analysis (Hernandez et al., 2004; ISO, 2005) and SIMQUANT analysis (Berdal et al., submitted). This ensures that PFU numbers can be compared directly since a non-biased ratio by default will be 1 : 1 : 1 : 1 : 1 for the five amplicons.

The material of the three non-GM maize lines was provided by Aust-Agder forsøksring, Norway, and consists of dried putatively homozygous leaves and sliced cobs with kernels harvested in the autumn 2004.

Four of the maize specific real-time PCR methods were developed and validated in the Qpcrgmofood project (Hernandez et al., 2004; Holst-Jensen, 2005). Notably, they differ in size from 79 to 136 bp in length and with the same template copy number in the PCR apparently do not produce identical Ct-values (Hernandez et al., 2004; Holst-Jensen, 2005). The validation of these methods was done according to the modularity principle (Holst-Jensen & Berdal, 2004), i.e. as stand-alone validation of the PCR method and one of these methods again validated by the CRL for Bt11 and T25. The fifth maize specific real-time PCR method was developed by a Japanese team and is an integral part of the Japanese standard for GMO testing and some of the annexed methods in EN ISO 21570 (ISO, 2005). The amplicon size for this is 151 bp. The method has been validated several times applying a global validation approach, i.e. the method has always been combined with a DNA extraction module and a GM specific real-time PCR module.

The size difference between the amplicons (79 – 151 bp) and the identical size of two of the amplicons (104 bp) could allow us to obtain indications also on the impact of amplicon size on sequence stability. Furthermore, the GC:AT bases pairs ratio is another factor where the study may provide some insight.

The starting point for the NVI was the establishment of calibration points of reference. For this purpose DNA was extracted with a CTAB method (CRL, 2005) from 6 sub-samples of the maize materials ground to flour immediately before DNA extraction. These sub-samples were selected as one leaf and one kernel sample per maize line.

The extracted DNA was diluted to a measured concentration of 40 ng/µl and further 4x diluted to assigned concentrations of 10 ng/µl, 2.5 ng/µl and 0.6 ng/µl, respectively. The six samples were successively analyzed by real-time PCR using the five methods described above and the resulting Ct-values for each combination of DNA extract, dilution and PCR method were tabulated.

The first crude visual inspection of the tabulated data did not find any indications of significant PCR inhibition in any of the DNA extracts (assessed by comparing ΔCt between the serially diluted reactions against the hypothetical value 2 for a 4x serial dilution).

Generally, the only clearly significant difference between the combinations was correlated to the PCR methods in question; the lowest Ct-values were observed for the *zein* method (average Ct for most concentrated was approximately 21.6), followed by *hmga* (22.2), *adh1* (23.0), *SSIIb* (24.0) and finally *inv* (25.3). There was no correlation observed between amplicon size and Ct-value, e.g. the targets for *zein* and *inv* are both 104 bp. For each method, the ΔCt value of the most concentrated and the most diluted sample should be 6 due to the dilution factor (64x dilution). The observed ΔCt for these materials vary between 5.1 and 6.9.

Based on the previous results where we observed variations in Ct between the different PCR methods, we decided to run SIMQUANT analysis on one material to find out whether the differences are caused by different copy numbers of the target genes or by the PCR method itself. Because we observed no significant variation between the different sample materials, we randomly selected the sample material to use for the SIMQUANT testing.

The results (see Berdal et al. [submitted] for details) clearly indicate that the observed variations in Ct-values in the initial experiment is NOT caused by varying copy numbers of the target genes but rather by the PCR assay itself.

The calibration points of reference are meant to allow us to compare data after processing with the reference, to determine:

a. The effect of processing on the general amplifiability/quantity of the DNA
b. If the amplifiability/quantity of any of the targets for the PCR methods, i.e. the analytes, is significantly more or less affected by the processing than any of the other analytes.

Processing may be applied to:

a. The original plant material, prior to DNA extraction
b. Extracted DNA

For the studies reported here, we chose to focus on the following types of processing:

I. Heating (physical degradation)
II. Modified pH (chemical degradation)
III. UV exposure (physical degradation)
IV. DNase treatment (enzymatic degradation)

First of all, we needed to establish appropriate protocols to ensure that degradation would be within a measurable range, preferably between 50% and 90% degradation over the exposure/time gradient. Real-time PCR methods are typically reported to show around 15-25% variation between replicates under within-laboratory reproducibility conditions.

A suitable protocol for performing acid treatment (modified pH, chemical degradation), UV based degradation (physical, example shown in **Figure 1**), DNase (enzymatic) and heat treatment (physical) degradation with the intended DNA degrading range have been developed and optimized within the first part of the project.

Except for the Dnase treatment experiments, we have obtained sufficient data to see certain patterns that will be described and discussed briefly in the following:

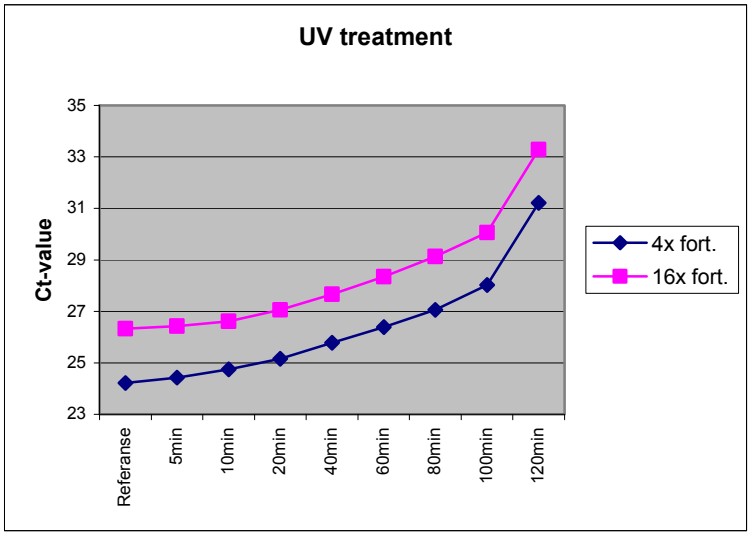

Figure 1. Example of a DNA degradation experiment with two samples analyzed with the *zein* method on DNA diluted 4 x and 16 x relative to the most concentrated template stock solution.

The heat-treatment experiments were designed to include two parallel series of the following materials:

- DNA extracted from 100 mg ground leaf material using the same CTAB extraction protocol that we use in the accredited GMO testing routine at the NVI.
- DNA extracted from 100 mg ground kernel material using the same CTAB extraction method as above.
- Ground leaf material (2 g) mixed in 20 ml MQ water in 50 ml Nunc tube
- Ground kernel material (2 g) mixed in 4 ml MQ water in 50 ml Nunc tube.

All the materials were subjected to heating at 100°C for 60 minutes and 120 minutes. The leaf and kernel materials were successively subjected to DNA extraction using the same CTAB extraction protocol as above. As reference, materials and DNA that had not been subjected to heating was used.

The UV experiments were performed with the following materials:

- DNA extracted from 100 mg ground leaf material using the same CTAB extraction protocol that we use in the accredited GMO testing routine at the NVI.
- DNA concentration was adjusted to 20ng/µl and each tube contained 50 µl (1µg DNA)
- DNA was aliquoted into PCR tubes places vertically in a rack and exposed to UV light for 10, 20, 40 and 60 minutes. As reference, untreated DNA (aliquots in PCR tubes) was used.

The pH experiments (depurination) were performed with the following materials:

- Citric acid as depurinating agent.
- DNA extracted from 100 mg ground leaf material using the same CTAB extraction protocol that we use in the accredited GMO testing routine at the NVI.
- A total of 2 µg DNA was incubated in a total volume of 500 µl at 37°C and 50 µl was taken out after 10, 20, 30, 40, 50 and 60 minutes and transferred to a clean tube.
- After transfer, the depurination reaction was stopped using TrisHCl (pH 8) and the tubes were successively stored at 4°C.
- DNA was then precipitated using EtOH and re-dissolved in 50 µl TE buffer.

After the treatments, DNA was analyzed by real-time PCR applying the five reference gene modules previously mentioned, with: 2 x, 8 x and 32 x dilutions, and the results averaged to estimate the PFU number in the undiluted DNA.

For the heat treatment experiment, the number of PFU was reduced to less than 0.1% of the original concentration for some of the combinations of treatment and amplicon. For the UV experiments, the number of PFU was reduced to approx. 0.1% of the original concentration for the most degraded combination, and for the depurination experiments, the number of PFU was reduced to approx. 6% or more of the original concentration.

A clear bias in degradation was observed, which to some extent can be correlated to amplicon size. However, we believe that the base composition may also explain a substantial part of the observed instability bias (**Fig. 2**). We are currently examining the data and designing new experiments that may add more data or confirm observed trends.

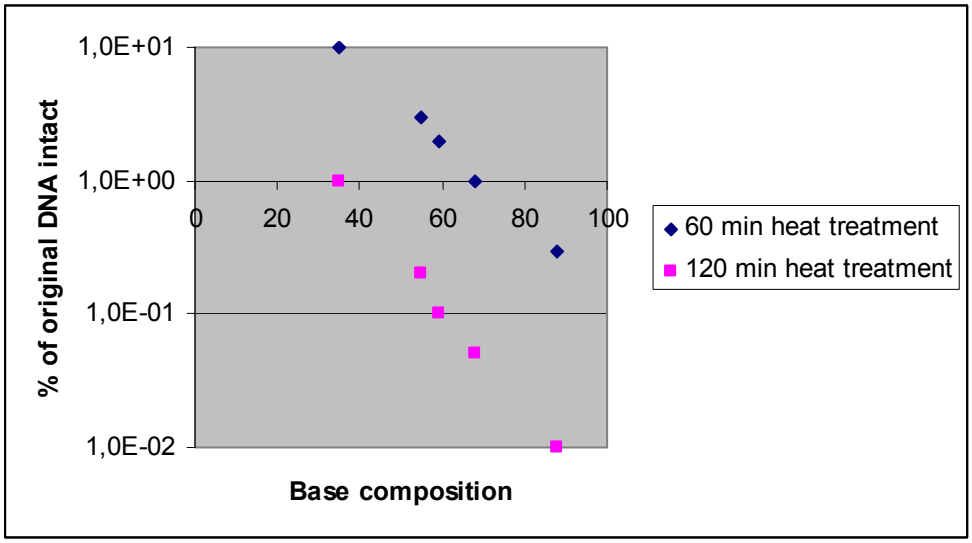

Figure 2. Example of biased effect on the number of PFU correlated with the base composition of the target amplicon. In this example the samples were heated at 100°C

3.2 CSIC, partner 21

The CSIC has focused on studies of the stability of the transgene at the level of DNA (work referred to WP5) and mainly (in WP6) at the level of RNA expression. In view of the GMO situation in Spain and in particular in Catalunya (i.e. 38,938 ha in 2005) Bt maize event Mon810 was selected as a model. The approach is based on the determination of possible variation among varieties and tissues.

Seeds were collected from a number of Mon810 varieties that are commercialized in Spain: the most frequently grown by farmers in the region are included; and so are the ones used in official evaluations of agronomic performance of varieties. Seeds from the following varieties are currently being assayed: *Aristis Bt, Asturial Bt, Bele Sur, Campero Bt, Coventry Bt, Cuartal Bt, DKC6041YG, DKC6575, Helen Bt, Jaral Bt, PR32P76, PR32R43, PR33P67 and Sancia Bt*. Grains of 12 of these varieties (obtained in the 2006 season) have been identified in agricultural fields and are in the process of collection.

As control, quantitative PCR reactions were performed with 100% GMO seeds of these varieties and no significant differences were detected (further assays with different GMO percentages (DNA mixtures) are currently being performed); and as expected, commercial *Cry* strip tests gave positive results for all varieties at the level of seeds and young leaves and grains.

This material is to be used in the collaboration between ILVO, CSIC (and NIB, partner 6, in connection with WP5) on target analyte stability over a wide range of Mon810 varieties provided by CSIC and NIB and analyzed by heteroduplex and capillary gel electrophoresis (CGE) optimized at ILVO. The objective is to detect and characterize sequence differences between Mon810 varieties at the level of maize genes, transgenic and flanking sequences (see also 3.3).

Regarding RNA expression, a duplex reverse transcription quantitative PCR assay was designed targeting the coding sequence of the Mon810 *cry* transgene and the maize nuclear small subunit (SSU) rRNA sequence as internal control. The assay uses the TaqMan® chemistry and the probes are labeled with FAM and VIC, respectively. The optimization of the duplex assay is performed using as template two *in vitro* transcribed RNA molecules corresponding to the *cry* transgene and the maize SSU rRNA. With uniplex reactions performing correctly, the duplex assay will be ready in the next 1-2 months.

In parallel, seeds of the Mon810 varieties have been grown under controlled conditions (*in vitro*) in order to compare the RNA levels of different varieties in the same conditions. Therefore, seeds, vegetative tissues and grains from different varieties will be initially compared (foreseen in the next few months).

Identification of fields growing Mon810 varieties and discussing with farmers has been achieved.

Conclusion

So far, the amount of data generated is limited. Future studies focusing on grain produced from seeds of these varieties, applying heteroduplex, CGE and transcription analyses are planned.

Note: we have collected the material and developed some of the PCRs to amplify various sequences of around 350-400 bp corresponding to transgene elements, flanking regions and also maize sequences. The heteroduplex analysis is planned to start at the end of November 2006.

3.3 ILVO, partner 22

The ILVO focuses on the nucleotide variability in the endogenous and transgene sequences. The experimental work plan has been set up with the following (internal to ILVO, not project specified) milestones:

- Experimental study on the nucleotide variability in endogenous and transgene sequences (Phase I, second half of second year)
- Approach to assess the influence of the nucleotide variability on the PCR efficiency (Phase II, end second year)
- Preparation of scientific publication on in field nucleotide variability of inserted and endogenous sequences (Phase III, third year)

The work is performed in close collaboration with CSIC in T6.6 and with NIB (partner 6, in WP5). The studies will be performed on maize, and the same five maize specific reference genes that are used by NVI (partner 7, see above) will be used in these studies. ILVO will in addition study the stability of the Mon810 insert in 14 Spanish varieties carrying this construct. The left and the right junction will be targeted as well the regulatory elements and the coding part of the construct will be analyzed. A set of 73 Slovenian wild type maize varieties, 14 Spanish maize varieties with introduced Mon810 construct and varieties from the variety bank of the JRC (partner 5) will be used. The nucleotide variability will be assessed by heteroduplex based method for mutation scanning - Conformation Sensitive Capillary Electrophoresis (CSCE), using a protocol optimized at ILVO (Papazova et al., in preparation).

Preliminary experimental data on the nucleotide variability of 5 endogenous sequences in 5 Belgian maize varieties and one maize inbred line are collected. The variability is in process of detailed characterization. Additionally, a system to evaluate the influence of the point mutations on the real-time PCR quantification is developed and tested. In this phase of the project DNA samples from 73 Slovenian varieties are received from NIB and will be screened by CSCE. In the coming months the analysis of the transgenic Mon810 sequences grown in Spain will start, part of which is foreseen to be performed in ILVO.

As soon as the complete set of samples (as described above) is received, analyses will be finalized and all data results will be processed and interpreted. The results will be described in a scientific publication in collaboration with the other partners.

3.4 GeneScan, partner 24

GeneScan has focused on a comparison of the effect on the estimated relative GMO content of GTS 40-3-2 (Roundup Ready) soybean (RRS) samples subjected to different types of processing, measured by application of three published combinations of reference gene and GMO specific PCR method.

3.4.1 Experimental design

The GM target of the first method (Berdal & Holst-Jensen, 2001) is event specific, while the other two (CODEX, 2001, ISO, 2005a) are construct specific and both target the junction between the 35S promoter and the EPSPS chloroplast transit peptide. All three methods use internal fragments of the soybean lectin gene *Le1* as reference target, but all three are distinct with respect to length, GC:AT ratio and theoretical T_m for the primers and probes. In other words, each method was composed of two "modules", a GM specific module (*x*, *y* or *z*) and a reference module (*X*, *Y* or *Z*), cf. Holst-Jensen & Berdal (2004). The GM quantity with each of the three methods was calculated as:

$$GM\%= \frac{x}{X}\times 100 \quad \text{or} \quad GM\% = \frac{y}{Y}\times 100 \quad \text{or} \quad GM\% = \frac{z}{Z}\times 100$$

The effect on GM% calculation if modules were substituted could also be studied by calculating the GM quantity as:

$$GM\%= \frac{x}{Y}\times 100 \quad \text{or} \quad GM\% = \frac{y}{Z}\times 100 \quad \text{or} \quad GM\% = \frac{z}{X}\times 100 \quad \text{or}$$

$$GM\%= \frac{x}{Z}\times 100 \quad \text{or} \quad GM\% = \frac{y}{X}\times 100 \quad \text{or} \quad GM\% = \frac{z}{Y}\times 100$$

Three different DNA extraction protocols (modules) were used, a CTAB/column based method (module α), a resin column based method (module β) and a more complex CTAB/spin-column method (module γ).

The material investigated was a 1% (weight:weight) "unprocessed" certified reference material (ERM-BF410d, formerly marketed as IRMM-410S) powder, a 1% (weight:weight) "processed" powder produced from a certified reference material (IRMM-410S-3) by Philippe Corbisier at the Institute for Reference Materials and Measurements, Geel, Belgium (JRC, partner 5 of the Co-Extra project). This powder has previously (Corbisier et al., 2005) been reported to exhibit degradation bias, as reported by INRA to the IRMM in 2000 (Bertheau personal communication). In addition, a 99.9% sample of GTS 40-3-2 was obtained from Brazil.

Total DNA mass determinations were done using a PicoGreen dsDNA Quantification kit (Molecular Probes). Conversion of mass determinations to target sequence copy number estimates was done on the basis of the haploid genome mass (1C) value reported for soybean by Arumuganathan and Earle (1991).

Calibration curves were produced using the estimated target sequence copy numbers on the basis of mass and genome size (1C value of soybean = 1.115 pg; Arumuganathan & Earle, 1991) to determine the copy number per calibration point. The material for calibration was produced by blending DNA from the 99.9% GM soybeans and non-GM soybeans, respectively, to produce a 10% GM-DNA:non-GM DNA mass ratio (5 x dilution series; 4 calibration points: 100 ng/reaction, 20 ng/reaction, 4 ng/reaction, 0.8 ng/reaction).

The first experiment focused on the influence of the DNA extraction method. To investigate the question whether the GMO percentage of a certain material, which is determined using quantitative real-time PCR methods, remains constant upon processing or exhibit biased DNA degradation, two different samples were analyzed: the 1% “unprocessed” RRS powder CRM and the 1% “processed” RRS powder which was produced from the former by a wet mixing process (Corbisier et al., 2005) as indicated in 2000 by INRA to IRMM (Bertheau, personal communication). These materials were chosen because (i) they were produced by the IRMM in Geel who probably has the broadest experience in the production of reference materials ensuring the absence of artifacts, e.g. due to lack of representativity; (ii) they have already been used in a similar study where significant differences in GMO% were reported (Corbisier et al., 2005); and (iii) 1% GMO represents a realistic contamination level and is close to the European labeling threshold of 0,9% (EC, 2003).

DNA was extracted from 100 mg aliquots with the three DNA extraction methods, with 10 replicates for each of the two samples and all three different methods, respectively. Subsequently the fragmentation state of the extracted DNA was evaluated by agarose gel electrophoresis and the concentration was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes).

3.4.2 Results

Extraction of DNA

The highest yield was observed for the α method. This is also the only method which performed very well on the “processed” RRS powder whereas especially the β but to some extent also the γ method yielded unsatisfying amounts of DNA. With the β protocol, no visible DNA could be extracted from the “processed” RRS powder. Therefore the extractions were repeated with a slightly modified protocol. Whereas with the γ method the DNA yield obtained from unprocessed and processed material was approximately the same, in case of method α a slight bias and in case of method β a strong bias was observed (method α: yield from “unprocessed” material >2 times higher than yield from “processed” material; method β: yield from “unprocessed” material >5 times higher than yield from “processed” material). Concerning the fragmentation state of the extracted DNA, no significant differences were visible in case of the “processed” sample material. However, in case of the “unprocessed” 1% RRS powder CRM some variation was observed: Whereas DNA extracted with method β was high molecular and formed a distinct band on the gel DNA extracted with the γ method showed a slight smear below the band of highly molecular DNA on the gel and DNA extracted with the α method displayed a smear from the top of the gel down to the bottom instead of any distinct band of high molecular weight DNA. Later DNA extractions from “unprocessed” 1% RRS powder CRM using the α method did not show this phenomenon. Therefore it may be that the observation is due to technical problems rather than inherent characteristics of the extraction method. This DNA was used in all further experiments described.

Quantification of Roundup Ready soy content

All 60 DNA extracts from “unprocessed” and “processed” RRS powder were analyzed using the three different real-time PCR methods. In case of DNA extracted using the α method 50 ng template DNA was used. Due to the low DNA yield encountered with method β and method γ in these cases only 10 ng template DNA could be used per reaction.

Estimates of the GMO content in the samples showed clear variation between the three real-time PCR methods, as well as between the “unprocessed” and “processed” sample.
The most pronounced differences were observed to be linked with one particular DNA extraction method and one PCR method. Quantification results for the 1% “unprocessed” RRS powder ranged from 0,96% (combination of modules *x*, *X* and α) to 1,90% (combination of the modules *y*, *Y* and β). Notably, the target copy numbers estimated on the basis of the calibration curves differed for all three RRS PCR modules (*x, y* and *z*), as well as for all three *Le1* PCR modules (*X, Y* and *Z*).

A comparison of results obtained with different modules was made, all using DNA extracted with extraction module α using 100 ng, 50 ng and 10 ng template DNA in triplicate, and a 10% calibrator prepared by mixing DNA from 99.9% RRS and non-GM soybeans. A clear difference was observed, clearly demonstrating the potential effect of combining biased PCR modules, see **Figure 3**.

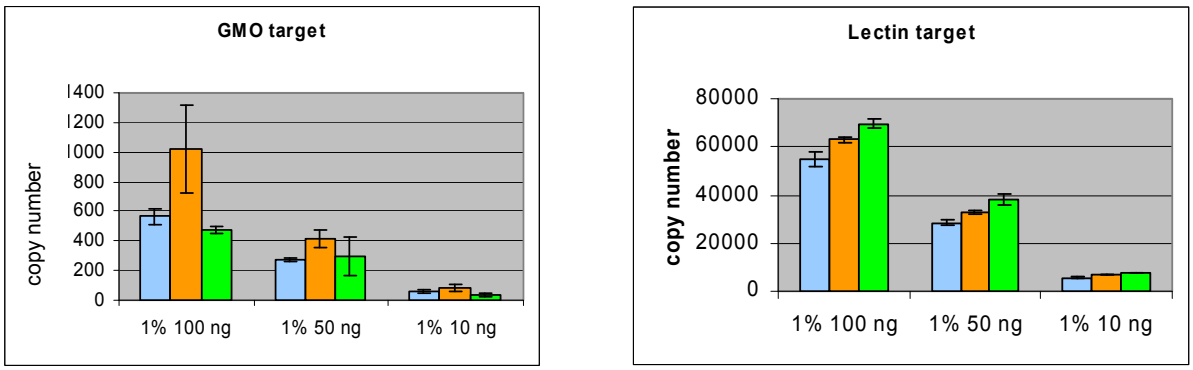

Figure 3. Lectin copy numbers and GMO target copy numbers determined with three different PCR systems. Depicted is the mean of six replicates respectively. The colors represent the following modules: blue = module z and Z, orange = x and X, green = y and Y, respectively.

Discrepancy between DNA quantification results using Picogreen and lectin copy numbers determined by real-time PCR

Although the concentrations of the DNA extracts obtained from 1% "unprocessed" RRS powder CRM and 1% "processed" RRS powder were carefully determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit, the lectin copy numbers determined by real-time PCR in some cases did not meet the expectations.

PicoGreen binds exclusively to double stranded and not to single stranded DNA. Consequently, a certain underestimation of the DNA concentration was expected, especially in case of the DNA extracts from the processed sample material. However, only with the γ method did we observe a higher lectin target copy number for the DNA extracts from the "processed" sample material compared to the DNA extracts from the "unprocessed" sample material. DNA extracts obtained with the β and α methods showed contrary effects.

Two other reasons for the discrepancy between DNA quantification results using Picogreen and lectin copy numbers determined by real-time PCR might be the unreliability of Picogreen DNA quantification because of interfering substances originating from the DNA extraction process or the sample matrix - or inaccurate lectin target quantifications because of substances influencing the PCR amplification kinetics. In case of the "processed" 1% RRS powder another possible explanation is differential analyte stability during the processing which might lead to under-representation or over-representation of certain target sequences in DNA extracts. Experiments were performed to assess these possible explanations, but without finding any evidence to support them.

Effect of processing steps on GMO quantification results

In some cases clear differences in quantification results of "unprocessed" and "processed" sample material were observed, e.g. for DNA extracted with the γ method the GMO content of the "unprocessed" sample was quantified to 1.45% whereas the GMO content of the "processed" sample was quantified to 0.84% using the same combination of PCR modules. A similar significant decrease in the measured GMO content was observed with another combination of PCR modules ("unprocessed": 1.08%; "processed": 0.55%). However there were also cases where the quantification results are almost perfectly identical (e. g. DNA extraction with module α, and the presumably best combination of PCR modules: "unprocessed": 0.96%, "processed": 0.95%; DNA extraction with module β, same PCR system: "unprocessed": 1.12%, "processed": 0.94%) - and there are in fact a lot of examples where certain differences in quantification results were observed. However, these

differences observed were not significant. In no single case did we observe a difference of quantification results for “unprocessed” and “processed” sample material to the extent described by Corbisier et al. (2005). And whereas in Corbisier et al. (2005) an increase in GMO% from “unprocessed” to “processed” sample material is described, we encountered rather a decrease and therefore a contrary effect – also when we used the same sample material, the same DNA extraction method and the same PCR system.

3.4.3 Discussion

Differential analyte degradation in processing steps

Taken together the data generated strongly suggest that there is no differential analyte degradation at least in a wet mixing process like the one which was applied to the 1% RRS powder CRM. Consequently the target ratio of transgene and reference gene remains constant during processing. Quantification results for the “processed” sample material which are in perfect accordance with quantification results for the “unprocessed” sample material demonstrate this. Furthermore, the diversity of the effects observed depending on the DNA extraction method and the PCR system also point strongly towards a more technical reason for the observed differences rather than a systematic bias in target analytes.

Extraction method dependent differences in the extractability of transgene and reference gene sequences as discussed in Corbisier et al. (2005) could explain why, in case of extraction with module α, no significant effects on quantification results were observed whereas when DNA was extracted with the γ module a lower GMO percentage was determined for the “processed” sample material than for the “unprocessed” sample material.

However, an alternative explanation could be partial degradation of double stranded DNA, different abilities to extract damaged and single stranded DNA, that differential PCR amplification rates/amplification kinetics occur due to soluble impurities in the DNA extracts or that DNA bound substances co-purified in the extraction process. Such factors could affect different PCR systems differently - and consequently could have an impact on quantification results.

3.4.4 Conclusion

Currently there are more questions than answers. Data evaluation is still going on and supplementary experiments will hopefully soon give some more insight into the problem.

3.4.5 Acknowledgements

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4 Summary of results, discussion and conclusions

The number of scientific publications reporting on possible bias in GM quantity estimations using real-time PCR based methods is growing, and can not be ignored. However, in most cases the experimental design does not permit clear conclusions to be drawn, because data produced could have several possible explanations. There are, however, a number of observations which crystallizes from these studies:

A. Size matters.
   a) When the GM and reference amplicon differ in length, the bias is pronounced in some cases where processing has a strong impact on the overall DNA degradation. The greater the size difference, the more bias towards shifting the ratio between the targets in favor of the smaller amplicon.
      In other words:
      - If the GM amplicon is shorter than the reference amplicon, then the GM content will be overestimated after processing.
      - If the GM amplicon is longer than the reference amplicon, then the GM content will be underestimated after processing.

      This observation is supported by published data by Moreano et al. (2005), (Yoshimura et al., 2005a; 2005b) and also by data generated by the National Veterinary Institute, Norway and GeneScan within task 6.6 of the Co-Extra project. See also review in Engel et al. (2006). Results published by Corbisier et al. (2005) have been slightly more unclear but could also be interpreted to support this observation.
   b) The relevance of particle size for each individual ingredient was pointed out by Moreano et al. (2005). When two different ingredients derived from the same plant species are mixed, the DNA degradation resulting from processing post-mixing may differ, e.g. as a result of differential exposure to degrading forces. Therefore, if the GM content of the two ingredients differs prior to mixing, a bias may be introduced.

B. The specific DNA extraction method (module) must fit for the specific matrix. An extraction module may be suitable for extraction of long DNA fragments but not for short fragments or it may be more or less able to remove inhibitory substances. Assessment of the quantity, quality and structural integrity of extracted DNA is crucial. This was stressed already by Holst-Jensen & Berdal (2004), and the importance was clearly demonstrated by Kobilinsky and Bertheau (2004) and Cankar et al. (2006).

C. Amplification efficiency must be assessed experimentally for the unknown sample, for both amplicons, and compared to the amplification efficiency for the calibration curves. Cankar et al. (2006) demonstrated that it is insufficient to assess the amplification efficiency by performance of a monitor run or use of an inhibition control amplicon. In their study they demonstrated that both amplicons could be affected without observable inhibition of the other, depending on yet undetermined factors. The most important criterion is that for a specific amplicon the amplification efficiency must be the same for the DNA from the unknown sample and for the calibrant DNA. Otherwise, the calculated PFU number for the unknown must be adjusted.

D. Structural composition of the amplicon and its flanking sequences may affect the availability of the sequence and its rate of degradation as a consequence of processing. This may include the base composition (as suggested by data from the NVI), as well as secondary folding structures and upstream and downstream regulatory elements as suggested by Nogva & Rudi (2004).

Future studies will try to quantitate the bias and develop guidelines to cope with this bias in the context of routine testing for GMOs.

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