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REVIEW

Relative quantification in seed GMO analysis: state of art and bottlenecks

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Abstract Reliable quantitative methods are needed to comply with current EU regulations on the mandatory labeling of genetically modified organisms (GMOs) and GMO-derived food and feed products with a minimum GMO content of 0.9 %. The implementation of EU Commission Recommendation 2004/787/EC on technical guidance for sampling and detection which meant as a helpful tool for the practical implementation of EC Regulation 1830/2003, which states that "the results of quantitative analysis should be expressed as the number of target DNA sequences per target taxon specific sequences calculated in terms of haploid genomes". This has led to an intense debate on the type of calibrator best suitable for GMO quantification. The main question addressed in this review is whether reference materials and calibrators should be matrix based or whether pure DNA analytes should be used for relative quantification in GMO analysis. The state of the art, including the advantages and drawbacks, of using DNA plasmid (compared to genomic DNA reference materials) as calibrators, is widely described. In addition, the influence of the genetic structure of seeds on real-time PCR quantitative results obtained for seed lots is discussed. The specific composition of a seed kernel, the mode of inheritance, and the ploidy level ensure that there is discordance between a GMO % expressed as a haploid genome equivalent and a GMO % based on numbers of seeds. This means that a threshold fixed as a percentage of seeds cannot be used as such for RT-PCR. All critical points that affect the expression of the GMO content in seeds are discussed in this paper.

Keywords Genetically modified organism · Quantification · Certified reference material · Plasmid · Real-time PCR · Calibrator

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Introduction

According to James (2012), 2011 was the 16th year of commercialization of biotech crops (1996–2011). Growth has continued after the first remarkable 15 consecutive years of increases, with a double-digit increase of 12 million hectares, at a growth rate of

8 %, reaching a record 160 million hectares (http://www.isaaa.org/). This has been accompanied by a considerable increase in the diversity of genetically modified organisms (GMOs) that have been approved worldwide. Recently introduced legislation [EU Regulation 1829/2003 (2003) and EU Regulation 1830/2003 (2003)] requires EU member states to test for GM presence in non-GM seeds and foodstuffs and enforce labeling when European Commission (EC) thresholds are exceeded. This legislation is likely to result in a rapid increase in the number of EU-authorized GM events; in addition, the number of world-wide GM events, as yet unauthorized in the EU, continues to grow (Ruttink et al. 2010). However, adequate detection tools are required to enforce these regulations, and this need has raised the issue of the adventitious presence of GM seeds in conventional seed lots. Adventitious presence may occur in all arable farming, at any step in the production of seeds or grain, or in processing of a harvested product in the food/feed chain. To date, there are no official thresholds governing the adventitious presence of GM seeds in conventional seed lots in Europe (Njontie et al. 2011). In this regard, the most accepted GMO detection methods are DNA amplification-based techniques, such as PCR (Hernandez et al. 2012), because they meet strict requirements, namely, high specificity, strong reproducibility, excellent efficiency and sensitivity (limit of detection and quantification; LOD and LOQ, respectively). The need for adequate reference standards is therefore twofold: firstly, qualitative PCR standards are required to act as positive controls for the identification of authorized and unauthorized GM events; secondly, quantitative standards are required to construct standard curves for GM DNA and total plant DNA to enable the absolute quantification of GM, thereby ensuring compliance with labeling requirements for authorized GM events (Allnutt et al. 2005).

A reference material for DNA-based methods is a material containing the analyte, and it can be a powdered material containing the analyte, DNA extracted from material containing the analyte, or a plasmid containing the nucleotide sequence of the specific analyte. Although certified reference materials (CRMs) should be used, if these are not available, a positive control sample can be used as reference material. Žel et al. (2008) stressed the point that a CRM for which sufficient information on the quality and origin is available is preferred to a reference material without a certificate or where the certificate is lacking essential information. More detailed technical provisions for the development and production and further requirements for reference materials, such as homogeneity, stability, storage, and certificate information, are described in Regulation (EC) 641/2004 (2004). Before plasmids can be used as reference material, the user must carefully ensure that the plasmid or the amplicon DNA sequence incorporated in the plasmid is suitable for the required purpose, namely, that the method to be used actually targets the sequence incorporated in the plasmid. One special topic that needs to be addressed in relation to reference materials and samples tested is that of biological factors. When dealing with plants, zygosity, tissue ploidy, and parental origin of the GM plant are important factors that can have an impact on the quantification of GMOs (Trifa and Zhang 2004; Zhang et al. 2008a, b) and which are discussed in this review. International trade requires reliable GMO analysis for comparable measurement of the GMO content in products (Trapmann et al. 2010). At both international and national levels the presence of GMOs should be expressed in percentages, but units are not specified. In the EU, Regulation (EC) No. 1830/2003 (2003) has not mentioned the measurement units for thresholds. In 2004, EC Recommendation 2004/787/EC proposed expressing "the percentage of genetically modified DNA copy number in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes." The lack of coherence between the legal requirement and possible methods for detecting GMOs has been described by Holst-Jensen et al. (2006). A different type of calibrant is needed for the GMO quantification result to be expressed in GMO mass fraction or GMO copy number ratio. It is recommended that CRMs prepared on a mass/mass scale and certified for the ratio between GMO and non-GMO counterparts of the same species be used for testing and the result expressed as the mass fraction. More information on both types of reference materials and their use in quality control and calibration can be found in different publications from the Institute of Reference Materials and Methods (IRMM), the Join Research Center (JRC), and the EC (Trapmann et al. 2010). Laboratories using PCR and quantitative (q) PCR can only measure DNA target copy numbers. Copy numbers are measured in CRM dilution series

using qPCR, and these values are used to build a calibration curve for determining the copy numbers in routine samples. Although sufficient information on CRMs is sometimes available for the transformation of mass/mass ratio to relative copy number (e.g., zygosity, tissue ploidy, parental origin of the GMO, extractability of DNA from material, among others), there is a lack of information on the composition of those samples tested. For these samples, there is obviously no information on the above-mentioned factors that influence GMO copy number.

In this review we have attempted to explain whether the influence of the genetic structure of seeds on real-time PCR quantitative results obtained for seed lots. The specific composition of a seed kernel, the mode of inheritance, and the ploidy level ensure that there is a lack of agreement between a GMO % expressed as genomes and a GMO % based on numbers of seeds. This means that a threshold fixed as a percentage of seeds cannot be used as such for real-time PCR. We also discuss the extent of these effects and the consequences if real-time PCR is used for the quantification of GM seeds.

Relative quantification in GMO analysis

Only qualitative PCR tests were possible for a long time, and these could only determine whether or not a sample contained the GMO. Quantitative tests that can reveal how much of a GMO is present are a relatively recent development. Only after the development of these procedures did the verification of thresholds become possible. However, even the very best techniques that are available at the present time are still prone to error. For the relative quantification of GMOs, two reactions must be performed on the same template DNA: one targets an endogenous gene that is specific to the plant taxon, and one targets a GMO-specific target. The relative percentage of GMO is obtained with two following methods:

1. Relative quantification using the 'delta CT method.' The objective of relative quantification is to compare relative amounts of nucleic acids among samples—and not to determine their absolute number (Ferré et al. 1994). Direct relative quantification of GMOs means that a relative percentage of GMO is directly obtained, without the need for absolute copy number quantification of the two targets. In fact, in the ΔCT method, both Ct values (of the reference gene target and the GMO target) are directly compared to each other. The difference between the Ct values (ΔCT) is used to directly calculate the GMO content:

$$\mathrm{GM}\,\% = (1/2\exp(\Delta\mathrm{ct})) \times 100\,\%.$$

2. Relative quantification using two absolute quantifications. Absolute quantification methods are used to determine the exact number of target molecules in a given sample (Ferré et al. 1994). In the 'standard curve method,' a standard curve is set up for each target, and CT values are calculated as a function of the absolute amount of the copy number of each target. Although based on two individual absolute quantifications, relative percentages are always needed for GMOs. In this case, comparison of copy numbers delivers a percentage of transgene target copies (genomes) relative to the total number of copies (genomes).

Direct comparison of two CT values (method 1) is only possible if the amplification efficiency of the two targets is exactly the same. As described above, the efficiency of the PCR assay depends, among others, on the quality, purity, and structural integrity of the template DNA. Comparison of two CT values is hampered if the two sequences are different in terms of detectability and/or quantifiability due to target sequence-specific effects, such as bias in the degradation of certain types of sequences (Holst-Jensen and Berdal 2004). Aguilera et al. (2009) used this method to determine the MON 810 insert copy number per haploid genome across 26 GM commercial maize varieties and found no significant differences between $2-\Delta\Delta$CT values respect to the standard curve values.

In the standard curve method (method 2), CT values are converted to initial target copy numbers. Unknown CT values are only compared to CT values of the same PCR product (of the standards) that has been amplified with the same efficiency (Holst-Jensen et al. 2003). The absolute standard curve method assumes that the amplification efficiencies of the standards and the unknowns are the same. Holst-Jensen and Berdal (2004) stated that another requirement for relative quantification is that the endogenous genes, to which comparison is made in relative GMO quantification,

should be consistently present in a single-copy/haploid genome, stable (i.e., without intra-specific allelic variation), and consistently distinct from any inter-specific counterpart.

State of art and bottlenecks for designing endogenous genes used for GMO quantification

Quantitative real-time PCR (qRT-PCR), the most widely used approach for quantifying the GM contents of GMOs, can be used to compare the copy number of the endogenous reference with that of the exogenous DNA fragment. The ideal endogenous reference gene should display species specificity, stable and low copy numbers in the genome, and low heterogeneity among different cultivars. Furthermore, the reliable RT-PCR assay of the endogenous reference gene should have a similar and high PCR amplification performance in different cultivars. Papazova et al. (2010) recently conducted a study on maize (*Zea mays*) in which they evaluated the reliability of eight existing maize reference assays, four of which are currently used in combination with an event-specific PCR assay that has been validated and published by the Community Reference Laboratory (CRL). These authors observed that assays targeting the zein and starch synthase (SSIIb) genes were highly reliable in terms of nucleotide stability and PCR performance, leading the authors to propose these genes as good alternative targets for a reference assay for maize. In the same context, Wang et al. (2010) compared the applicability of four endogenous rice reference genes, including sucrose phosphate synthase (SPS), GOS9, phospholipase D (PLD), and ppi phosphofructokinase (ppi-PPF), in quantitative GMO analysis. Their study demonstrated that the SPS and ppi-PPF quantitative PCR systems were applicable for use in a rice endogenous reference assay, with less variation among the Ct values, good reproducibility in quantitative assays, and low M values by comprehensive qPCR comparison and GeNorm analysis. Mbongolo Mbella et al. (2010) designed a set of SYBR®_Green qRT methods for the detection of endogenous reference genes in commodity crops. These studies are of major importance in that they identify reliable reference genes to be used in quantitative analysis in routine laboratories. Table 1 describes the endogenous genes used for GMO quantification. Chaouachi et al. (2007) that a good knowledge of the species under consideration is necessary when determining the bottlenecks in any reference system for GMO application. For each plant species targeted, a study of its taxonomy allows the researcher to choose certain plants that are presumably the most representative of the relationships to the considered, i.e., wild-type plants, and also to choose the most commonly cultivated species. Another point to be considered is the availability of the DNA sequences and the sequencing procedure to be applied using the lines and cultivars of the taxa to be studied. This will lead to the detection of the eventual polymorphism, such as the single nucleotide polymorphism (SNP), and to avoid false negatives. Wang et al. (2010) detected SNPs in the target regions of four endogenous reference genes among 58 rice cultivars, all in GOS9 genes and the PLD target region. It is also crucial to have an understanding of the possible development of hybrids through interspecific and intergenic crosses with related species as such hybrids could result in the introgression of novel traits into this related species. Three cases of the introgression on novel traits have been the subject of extensive discussion:

Case 1. Phylogenetically related species: example of potato (*Solanum tuberosum*) and tomato (*S. lycopersicum*);
Case 2. Occurrence of introgression during plant breeding: example of sugar beet (*Beta vulgaris*);
Case 3. Influence of ploidy level: example of rapeseed (*Brassica napus*).

Therefore, there is a strong necessity to have an international collection called "*core collection*" for the validation of the methods. Such information will allow the researcher to enumerate the species required for the specificity test and, as a consequence, avoid discordance of results when different plant material in a different geographic area is used (Chaouachi et al. 2007).

Measurement and expression units for GMO contents

DNA quantification as such is very difficult because only relative data can be obtained. Two absolute numbers of DNA sequences cannot be compared directly to each other, but must be normalized to some

Table 1 Evolution of expression units of genetically modified organism (GMO) content stipulated in EU legislation on GMOs

Evolution EU regulations	Units of GMO expression	
	Food and feed	Seeds
2000		
EC/2000/49	% Mass/mass?	% Seeds/seeds?
EC/2000/50		
2004		
2003/1829/EC	% HGE/HGE?	% Seeds/seeds?
2004/787/EC		

entity. When working with genome/genome (g/g) ratios, determination of the number of copies of both an endogenous target and a GMO-specific target gene assumes that equal amounts of DNA have been used for both targets. The main difficulty of DNA quantification is linked to the measurement units. In RT-PCR, which is the accepted reference technique for GMO quantification in the EU, a fluorescent signal is measured. This signal is converted to a concentration or amount of initial target, depending on the unit of the calibrators used. Commission Recommendation 2004/787/EC (2004) on technical guidance for sampling and detection, which was set up as a helpful tool for practical implementation of Regulation 1830/2003 (2003), states that 'the results of quantitative analysis should be expressed as the number of target DNA sequences per target taxon specific sequences calculated in terms of haploid genomes'. With this recommendation, the use of pure DNA calibrators, expressed in copy numbers of target sequences, is recognized. This unit and this type of calibrator are in line with the concept of modularity of the GMO analytical procedure and validation approaches (Holst-Jensen and Berdal 2004). Considering the genome/genome ratio as the unit of measurement in PCR-based quantitative methods for GMOs sounds logical—but this is not completely correct. Due to the above-mentioned processing-, matrix- or extraction-based effects of degradation and inhibition of nucleic acids, target copies may be damaged or for a number of reasons fail to act as templates for PCR. Because the number of target copies assumed to be present in the reaction tube—and to be amplified—is not necessarily equal to the number of copies actually measured, Holst-Jensen and Berdal (2004) stated that 'PCR-forming units' (PFUs) are the only correct units of measurement. Before the two new regulations came into force (April 2004), the situation was much more complicated as there used to be a gap between the analytical unit of measurement (amplifiable DNA target copies or PFUs) and the legal unit of measurement (e.g., mass of material). Early European legislation [Directives (EC) 49/2000 and (EC) 50/2000)] required the percentages of GMOs but never specified the basis of this percentage (Kay and Van den Eede 2001). From the beginning, it has been clear that quantification must be relative to the ingredient, but this could be either on a weight basis, an amount of matrix, or a number of units (particles such as grains/seeds, but also DNA/protein molecules). Based on early labeling regulations, any unit could thus be used. However, as early as in the 1990s, GM contamination levels in different types of samples were determined by means of RT-PCR using CRMs with mass GMO percentages as calibrators. As a consequence, quantitative results from RT-PCR assays were expressed also in mass percentages. Because powder CRMs were the only reference calibrators available at that time, a mass ratio was assumed to be the unit for measurement of GMO contents (Table 2).

This necessitated a translation from what was being measured at the DNA level to any other unit used for expressing the GM contamination, such as the percentage of mass or number of particles. The discordance, however, was that there is no exact relationship established between a mass or number of grains and a number of amplifiable DNA molecules; consequently, this translation cannot be made. The disagreement between the different units for GMO expression is of crucial importance on quantification and has influenced the implementation of GMO legislation. Therefore, this major difficulty will be described more extensively. Two kinds of discussions can be pursued in the framework of GMO expression units and quantification, as illustrated in Fig. 1.

Units of measurement: example of the effect of the genetics of plant seeds and the relative parent contributions

Although not specified, the unit of expression stipulated in early threshold regulations was assumed to be

Table 2 Influence of the genome size and the amount of DNA used on the detectability and quantifiability of transgenic DNA in PCR assays

Species	Family	Ploïdy level (*n*)	DNA amount 1C value (pg)[a]					1C (Mbp)[c] theoretical	LOD (%GMO)[d]
			Cmin	Cmax	Cmean	SD	PRIME value[b]		
Arabidopsis thaliana (L.)	Cruciferae	2	0.05 (Francis et al. 1990)	0.45 (Schmuths et al. 2004)	0.19	0.09	0.16 (Bennett and Smith 1976)	158	0.00016
Maize (*Zea mays*)	Gramineae	2	2.35 (Bennett and Smith 1976)	6.30 (Hake and Walbot 1980)	2.97	0.88	2.73 (Bennett and Smith 1976)	2,694	0.0027
Soybean (*Glycine max*)	Leguminosae	2	0.93 (Doerschug et al. 1978)	1.98 Greilhuber and Obermayer (personal communication, 1996)	1.29	0.5	1.13 (Goldberg 1978)	1,115	0.0011
Canola (*Brassica napus*)	Cruciferae	2	1.15 (Greilhuber 1988)	1.60 (Nagl et al. 1983)	1.38	0.49	1.15 (Greilhuber 1988)	1,135	0.0011
Cotton (*Gossypium hursitum*)	Malaacea	4	1.6 (Walbot and Dure 1976)	3.23 (Bennett et al. 1982)	2.66	0.55	2.4 (Hendrix and Stewart 2005)	2,369	0.0024
Sugar beet (*Beta vulgaris* L.)	Chenopodiaceae	2	0.75 (Ingle and Sinclair (1972)	1.30 (Ingle and Sinclair 1972)	0.98	0.22	1.25 (Bennett 1972)	1,234	0.0012
Tomato (*Solanum lycopersicum*)	Solanaceae	2	0.75 (Galbraith et al. 1983)	2.55 (Van't 1965)	1.20	0.52	0.75 (Bennett and Smith 1976)	740	0.00075
Wheat (*Triticum aestivum*)	Gramineae	6	15.70 (Ingle and Sinclair 1972)	19.45 (Hülgenhof et al. 1988)	17.69	0.87	17.33 (Bennett and Smith 1976)	17,109	0.017
Barley (*Hordeum vulgare* L.)	Gramineae	2	3.63 (Marie and Brown 1993)	5.85 (Hülgenhof et al. 1988)	5.13	0.5	5.55 (Bennett and Smith 1976)	5,480	0.0055
Tobacco (*Nicotiana tabacum* L.)	Solanaceae	4	3.30 (Zimmerman and Goldberg 1977)	7.70 (Nagl et al. 1983)	4.85	1.96	5.18 (Leitch et al. 2008)	5,114	0.0051

Cmin Minimum value of C, *Cmax* maximum value of C, *SD* standard deviation

[a] PRIME value: several estimates for a species have been published or communicated, then one PRIME estimate has been identified as the estimate preferred to any other for that species

[b] Haploid genome size, calculated with the formula $n = m \times 986.9$ where n is the number of base pairs (Mbp) and m is the amount of DNA in pg (as listed in Database of Genome Sizes; DOGS)

[c] Number of haploid genome copies = haploid genome equivalents

[d] The theoretical limit of detection (LOD; Bennett and Leitch 2010) is 1 copy of the haploid genome

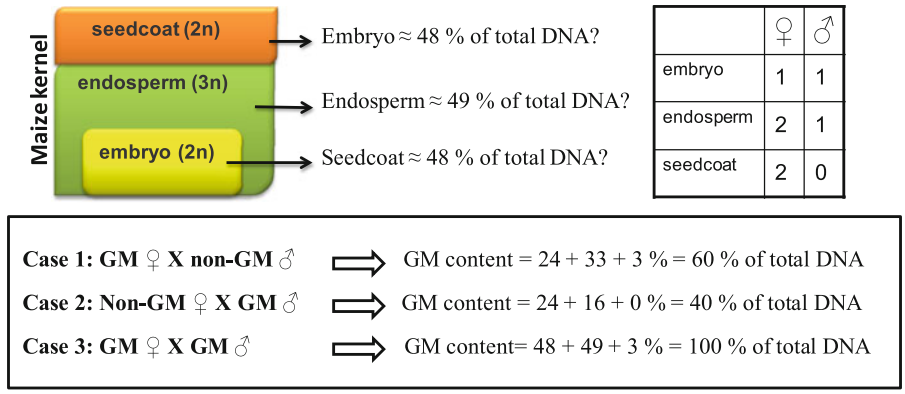

Fig. 1 The effect of ploidy level and parental contributions on the units of measurement. Example of maize (*Zea mays*). Three cases are described depending on the parental contribution and the ploidy level of the kernel composition. The results demonstrate the differences observed in the genetically manipulated organism (GMO) content of the total DNA. *Case 1* is the result of a cross between a GM female and non-GM male; the final GMO content is about 60 %. *Case 2* is the result of inbreeding between a non-GM female and a GM male; the final GM content is about 40 %. *Case 3* describes inbreeding between a GM male and a GM female; the final GM content is 100 %. These cases lead to the conclusion that three parameters may be considered by the competent authorities: (1) parental contribution, (2) ploidy level; (3) species to be tested. These parameters directly influence the GMO content quantified by the quantitative real-time (qRT)- PCR assay that is considered to be the reference method in this field

based on weights (mass) of material. GMO estimates have generally been based on the assumption that the genome/genome (g/g) ratio is equal to the weight/weight (w/w) ratio. However, this is only the case if the GMO material has been submitted to exactly the same treatment as the non-GMO material. In other words, taking the relation between two amounts of measured DNA is only possible if the number of DNA molecules obtained from one weight unit or grain is exactly the same regardless of the origin. Theoretically, it cannot be excluded that, for the same weight of material, different amounts of DNA are extracted from a transgenic seed and a conventional non-GM seed respectively. Mano et al. (2009) recently designed and validated a new strategy for evaluating the w/w GMO content in maize grains. It should be noted that because of the increasing use of maize hybrids with GM stacked events, the established and commonly used bulk sample methods for PCR quantification of GM maize in non-GM maize are prone to overestimate the content of the GMO compared to the actual w/w percentage of GM maize in the grain sample. The newly developed method of Mano et al. (2009) is a PCR-based qualitative analytical method consisting of a sample preparation step in which 20 maize kernels are ground in a lysis buffer and a subsequent PCR assay in which the lysate is directly used as a DNA template (Mano et al. 2009). Trifa and Zhang (2004) provided a good example of the difference between the units of expression of GMO content in seeds. It is known that the different maize kernel cultivars, including transgenic ones, contain different DNA amounts per mass unit. In a certain maize mixture, the DNA ratio of a certain cultivar is not necessarily proportional to the weight ratio of that cultivar in the mixture. A maize kernel consists of an embryo (12 %), an endosperm (82 %), and a seed coat or tegument, including pericarp (6 %). Seeds are formed through double fusion of nuclei. First, a haploid maternal nucleus and a haploid male nucleus fuse to form the diploid embryo (2*n*). Second, the triploid (3*n*) endosperm results from the fusion of two material polar nuclei with one sperm nucleus. Intensive mitosis, followed by endoreduplication of the DNA, results in endosperm development. Embryo and endosperm are enveloped by a diploid tegument (2*n*), originating from the mother only (Lopes and Larkins 1993; Schweizer et al. 1995; Trifa and Zhang 2004). Transgenic maize kernels are hemizygous, resulting from the crossing of a homozygous transgenic parent with a non-transgenic one. Because male and female genomes do not equally contribute to the maize kernel, the relative content of a transgene genome in heterozygotes will be dependent on inheritance. This effect would be negligible if one could assume that the kernel's total DNA mainly originates from the embryo

and if only this embryo part would be used for DNA extraction. However, as whole seeds are milled and used in DNA extraction and as endosperm cells can contain up to 690C—with C being the unreplicated, haploid DNA content per nucleus (Kowles and Phillips 1985)—this is not the case. Papazova et al. (2005) experimentally determined that, in the case of maize, the embryo's DNA content per mass unit is about twice that of the endosperm. This principle is illustrated in Fig. 2, which shows different ploidy levels of three plant families [Gramineae maize (*Zea mays*), Chenopodiaceae (*beet*), and Fabaceae (soybean (*Glycine max*)] and the effect of plant seed genetics and the relative parent contributions. Figure also 2 describes the case of maize (*Z. mays*) as an example of the discordance between parental contributions and GMO content. Such differences in DNA content per weight unit between cultivars and between GM and non-GM grains will result in differences between w/w (e.g., flour from whole grains or beans) percentages and measured g/g (amplifiable DNA molecules) percentages. (Anklam et al. 2002; Holst-Jensen et al. 2003). More recently, Liu et al. (2010) analyzed whole-seed DNA density and DNA proportions in the hull, endosperm, and embryo of rice seeds collected from 19 different cultivars (of different rice species) and established two equations to estimate the GM content in the rice samples that took into account the influence of the different tissue DNA content of the rice seeds during GM rice seed quantification. The GM_{hg} percentage of the sample containing hemizygous GM rice seeds can be calculated using the equations shown below.

In the case of the GM allele from the female parent:

$$GM_{hg}\% = GM_{wt}\% \times (X + 2/3 \times Y + 1/2 \times Z). \quad (1)$$

In the case of GM allele from the male parent:

$$GM_{hg}\% = GM_{wt}\% \times (1/3 \times Y + 1/2 \times Z) \quad (2)$$

where X, Y, and Z represent the average percentage of the hull, endosperm, and embryo DNA to total DNA, respectively. According to Liu et al. (2010), these two proposed equations reduce the bias of quantifying GM

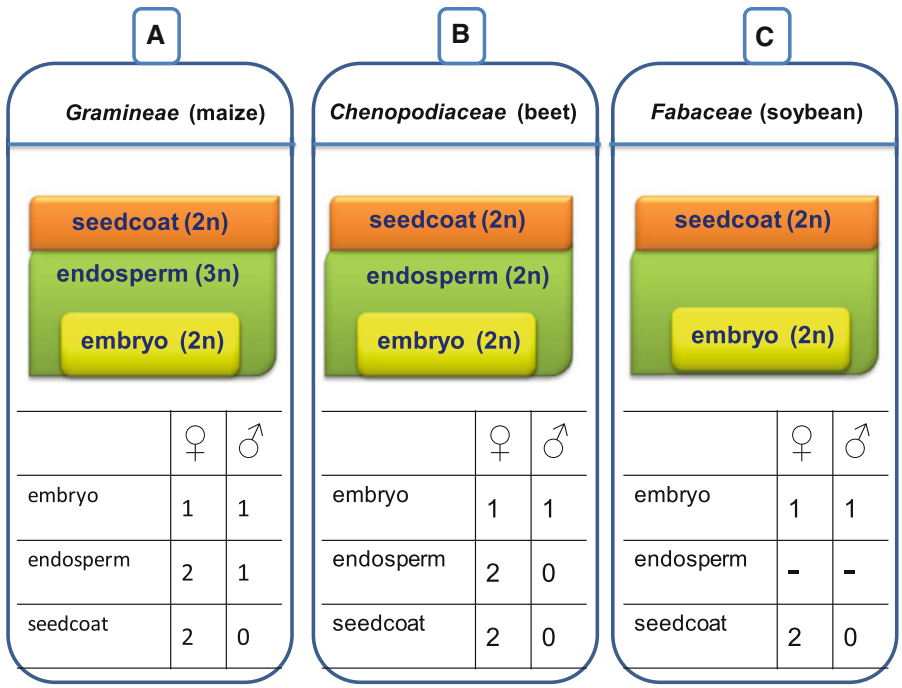

Fig. 2 Effect of plant seed genetics and the relative parental contributions on the expression units of measurement. Three plant families are cited depending on the ploidy of seed components (seedcoat, endosperm, and embryo). **a** Case of the Gramineae family (e.g., maize *Z. mays*), **b** Chenopodiaceae (e.g., sugar beet (*Beta vulgaris*), **c** Fabaceae (e.g., soybean *Glycine max*). These three families are different not only in terms of endosperm ploidy level, but also in parental contribution

rice seed and grain production when the analyte or reference materials contain heterozygous GM rice seed powder. The same approach was used by Liu et al. (2010) to experimentally assess the impact of biological factors on quantitative results, analyzing by qRT-PCR six maize MON 810 hybrid kernels with different genetic structures: (1) hemizygous from transgenic male parent, (2) hemizygous from transgenic female parent, and (3) homozygous at the transgenic locus. The results obtained in this study showed clear influences of biological factors on GM DNA quantification: 1 % of GM materials by weight for the three genetic structures contained 0.39, 0.55, and 1.0 % of GM DNA by HG, respectively, as determined from qRT-PCR analyses. This finding can be used by stakeholders for empirical prediction from one unit of expression to another in the monitoring of seed and grain production chains in GMOs (Liu et al. 2010).

According to the results cited above, quantification of GM DNA in seeds is dependent of the origin of the seed, particularly that of the transgenic parent, and thus no relationship can be determined based on a certain genome ratio to a single value weight ratio, or vice versa. It is therefore difficult for the competent authorities to interpret the final (converted) result. In addition, the operational procedure from sampling to the application of the qualitative or the quantitative method is crucial for the determination of GMO content in seeds. One of the major step in this procedure is grinding to facilitate DNA extraction. Combining the ploidy level of the seed composition before and after grinding can lead to errors in GMO quantification. As an example, Fig. 3 shows the effect of grinding on the expression units of GMO content in the case of beet seeds. In fact, before grinding all seeds have a GM embryo and are homogeneous; however after grinding the sample becomes heterogenous (embryo-derived particles = GM endosperm-derived particles = non-GM).

In conclusion and despite the fact that controversies and debates are on-going and no specified legislation has been established for the labeling and analysis of transgenic seeds, it is generally assumed that the GM content in seed lots should be expressed on a seed-to-seed basis. If a seed/seed percentage is to be the unit for expressing GM contamination levels in seed samples, one can immediately conclude that RT-PCR is not the suitable technique for quantification based on the specific nature and structure of a seed.

GM contamination levels expressed on a haploid genome basis

European Commission Recommendation 2004/787/EC (2004) states that results should be expressed in terms of DNA copy numbers. This recommendation is the result of many fruitful discussions and positive developments carried out within the ENGL Working

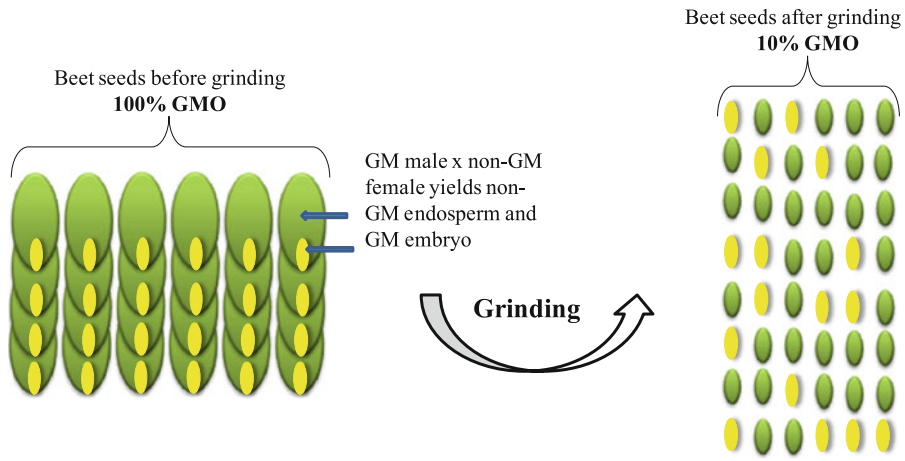

Fig. 3 Effect of grinding on the units of measurements and expression of GMO content in presence of seed matrix. Example of beet seeds to be tested. Beet seeds obtained from crossing a GM male and non-GM female are used as an example. Before grinding, all seeds have a GM embryo and are homogeneous; however, after grinding the sample becomes heterogenous. (embryo-derived particles = GM endosperm-derived particles = non-GM)

Group 'Thresholds'. In this context, Patricia Bonner from the ENGL proposed the concept that a conversion factor (Cf1) was needed to link the weight% GM of the used calibrant with the copy number ratio estimated for the sample, which is the output of the test method. A second conversion factor (Cf2) would then be required to convert the copy number ratio to the final expressed weight% of GMO. This proposal has finally led to the acceptance by the EC of the new definition of a GM percentage in terms of haploid genomes. The EC's viewpoint is that this new definition should be used along the food and feed chain and should also be reflected in the seed regulations that are currently under development. It is also agreed that no translation to any other unit of expression should be made, as conversion factors are extremely difficult to estimate and would require recalculation for each type of material.

The new accepted unit for measurement and expression of GMO content is the most coherent approach, as it solves many problems. If reference materials are used that allow the GMO content to be quantified and calculated in terms of haploid genomes, and if no further translation to %GM material is needed, then the earlier mentioned biological factors no longer present a problem. The DNA copy number unit is the only correct, indisputable, and unambiguous way of expressing a measured content of GMO. It will clear away possible misinterpretations and ensure that results on the same sample, obtained by different laboratories, are comparable. This requires the use of a universal type of DNA calibrants with certified copy number values.

Use of plasmids as calibrants in GMO quantification

As pure analyte standards, the choice can be made between genomic and plasmid DNA. Genomic DNA needs to be extracted from a matrix first and thus is susceptible to matrix effects and processing influences, such as degradation. Plasmid DNA vectors that contain the sequence(s) of interest are easier to handle because of their simple and cheap production process, their stability, and their universality and wide applicability. Four classes of plasmid are necessary for GM event identification and quantification:

1. Generic GM sequence plasmids containing a commonly used GM sequence, such as the 35S promoter. Such plasmids can be used as standards across different species and events.
2. Event-specific plasmids containing a unique sequence which flanks a GM event and plant DNA insertion site or a unique sequence within the GM event. At least one of this class will be required for every GM event to be studied.
3. Construct-specific plasmids containing sequences of junctions between two markers interior to the insert, such as the junction P35S/bar in the Bt176 event.
4. Endogenous control plasmids containing a specific (but not necessarily exclusive) sequence of the target species' nuclear genome, which is present in a known, low-copy number. These plasmids are essential to allow the absolute quantification of the number of GM events present per total number of plant genomes and, therefore, the %GM DNA. One plasmid of this class is required for each crop species.

The calibrants to be used as reference material for relative quantification have been the subject of many European projects since 2001. The main project cited is entitled the "Tracing and authentication of GMOs and derived products in the food-processing area" [Scientific support plan for a sustainable development policy (SPSD II), January 2005)] and included many partners (Institute of Public Health (ISP); Centrum voor Landbouwkundig Onderzoek (CLO), among others] as the construction of a plasmid bank has been enlarged to other non-financed partners, including the Centre de Recherches Agronomiques (CRA), the Institut Scientifique de Recherche Agronomique (INRA; Versailles, France), the Institute for Health and Food Protection (IHFP; Ispra, Italy), the Institute for Reference Materials and Measurements (IRMM; Geel, Belgium), and the Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes (DGCCRF; Strasbourg, France). At the end of the project, the number of constructed plasmid had increased to 151, including the three GMO markers of different GMO events (event specific, construct specific, and screening) and also different species with the respective endogenous genes (details and tables mentioned in the report of the project CP32, available at: http://www.belspo.be/). Two kinds of plasmids were constructed, the single-target

plasmid (STP) and the multiple target plasmid (MTP), containing one or many GMO markers respectively. In addition an ENGL plasmid database was constructed and is not yet publicly accessed in the web. In fact, all the constructed pENGL are deposited in the official plasmid and cDNA collection (LMBP) from the Belgian Coordinated Collection of Micro-organisms (BCCMTM) of Gent (Belgium) as central master depository under the statute of full private deposit. According to the literature, many STP or MTP plasmid calibrants were also constructed and validated. For example Lievens et al. (2010) have constructed and assessed a dual-target plasmid, designated as pJA-NUS^{TM}-02-001, comprising part of a junction region of genetically modified soybean event GTS-40-3-2 and the endogenous soybean specific lectin gene was constructed. Zhang et al. (2008a, b) developed one unique plasmid molecule based on one pMD-18T vector with three exogenous target DNA fragments of Roundup Ready soybean GTS 40-3-2 (RRS), that is, CaMV35S, NOS, and RRS event fragments, plus one fragment of soybean endogenous Lectin gene. Moreover, Yang et al. (2007) have developed one novel standard reference molecule containing seven GM maize events (Bt11, Bt176, GA21, MON810, MON863, NK603, and T25). All nine specific integration junction sequences of these GM maizes and the maize endogenous reference gene, zSSIIb, was constructed and used for quantitative analysis. As second type of DNA calibrators another plasmid was constructed by Kuribara et al. (2002) and validated in an interlaboratory trial by Shindo et al. (2002). The plasmid contains a DNA sequence of a region specific for event 40-3-2 (Roundup Ready soybean), sequences of the CaMV p-35S and *Agrobacterium tumefaciens* t-NOS, as well as a lectin (*Le1*) gene sequence specific for soybean, in one and the same vector. The product is linearized DNA, digested with a restriction enzyme and diluted with salmon sperm DNA (Kuribara et al., 2002). Taverniers et al. (2004) have described two QRT-PCR methods for Roundup Ready soybean used for relative quantification, in which two types of plasmid DNA fragments were used as calibrators. Single-target plasmids (STPs) diluted in a background of genomic DNA were used in the first method and Multiple-target plasmids (MTPs) containing both sequences in one molecule were used as calibrators for the second method. Both methods simultaneously detect a promoter 35S sequence as GMO-specific target and a *lectin* gene sequence as endogenous reference target in a duplex PCR. In the other hand, Taverniers et al. (2005) have also constructed event-specific plasmid standards for the events Bt11, Bt176, and GA21 maize and the event GT73 canola. In 2007, Dalla Costa et al. (2007) have developed new MTP pGEM-T Plasmids containing *adh1* − *lect1* and *zein* − *lect1* endogeneous genes and found to be the most reliable calibration systems for this analysis, providing precise and accurate quantification results. In 2011, Li et al. (2011) have developed a flexible plasmid RM pNK containing three DNA fragments, namely the 5′ and 3′ event-specific sequences of maize NK603 and endogenous gene *zSSIIb* and proved the suitability of this calibrator compared with that of genuine genomic DNA. Another recent study conducted by Khoo et al. (2011) has developed a new recombinant plasmid DNA used as calibrator for the quantification of the unapproved Starlink corn and the approved Bt176 maize present in the maize containing foods, such as raw maize and processed food, as well as, animal feed in Malaysian market. Finally, Meng et al. (2012) have reported the construction of plasmid pTC1507 for a quantification assay of the maize event TC1507 and the collaborative ring trial in international validation of its applicability as a plasmid calibrant. pTC1507 contained one event-specific sequence of TC1507 maize and one unique sequence of maize endogenous gene zSSIIb. Mattarucchi et al. (2005) have constructed and used recently a tandem marker plasmid as a competitor for the detection and quantification of genetically modified cotton MON-531. This plasmid contained event specific sequence of GM Bt cotton MON-531 and taxon specific sequence of cotton that is fsACP (fiber specific acyl carrier protein). More over comparison studies were conducted to search the suitability of two types of DNA calibrants, i.e. plasmid DNA and genomic DNA extracted from plant leaves, for the certification of the GMO content in reference materials as copy number ratio between two targeted DNA sequences was investigated. Caprioara-Buda et al. (2007) have observed that both plasmid and leaf genomic DNA calibrants would be technically suitable as anchor points for the calibration of the real-time PCR methods applied in GMO analysis.

Genome-related problems in GMO quantification

Quantitative results from protein-based analyses are expressed on a w/w basis, while quantitative results

from DNA assays are expressed in terms of genome equivalents. As a consequence, DNA quantification and, in particular, the limits of detection and quantification are influenced by the number of copies of the gene present in the test sample—and thus by genome size (Anklam and Neumann 2002). As reported in the literature, the nuclear genome size varies considerably between angiosperm species. The amount of DNA in the unreplicated haploid nuclear genome of an organism is referred to as its *C*-value and expressed in picograms (pg) of DNA or in megabase (Mb) pairs of nucleotides. Table 2 shows the relationship between the genome size of different genetically transformed species (1C value), ploidy level, absolute amount of target sequence corresponding with threshold percentages for GMOs, and the theoretical limit of detection (LOD). As shown in Table 2, the larger the genome size, the smaller the number of copies present in a fixed amount of DNA (typically 100 ng for PCR reactions) and, therefore, the smaller the number of GM molecules present in a 0.1 % GMO sample. According to the DOGS database and results obtained from the literature, there is discordance among results of genome quantification, depending on the method used. For example, for maize the C value ranged from 2.35 (Cmin) to 6.30 (Cmax) and the PRIME value used by the scientific community is 2.73. This difference can lead to errors in LOD estimations. In fact, the chance of detecting one single copy of the haploid genome, corresponding with the theoretical LOD, is different for each species, as the PCR amplification efficiency is influenced by the total amount of DNA present in the reaction. For GM soybean, 1 GM copy corresponds to 0.001 %, while for GM maize, this value is 0.003 %. The LOD and quantification are always related to the genome size of the species under study, but also to the amount of DNA used and, therefore, to the analyzed test portion and the original sample size (Kay and Van den Eede 2001; Van den Eede et al. 2002). Roldan-Ruiz et al. (2001) showed that the LOD and quantification, and qPCR results in general, based on genome copy numbers, must always be expressed with respect to the total amount of DNA present in the reaction tube. Genome-related factors, such as ploidy and zygosity levels, have become irrelevant based on the use of the haploid genome copy number unit for the expression of GMO contents,. However, this DNA-based unit per definition only allows the researcher to make a statement on the presence/content of a certain genetic element. For example, if relative quantification is based on the presence of p-35S, the result merely relates to this genetic element and does not allow any conclusion to be drawn on the presence of different GM events. The identification of biological variation between the original, 'approved' variety and other, 'cultivated' varieties also needs other approaches (Van den Eede et al. 2002; Van Duijn et al. 2002; Wiseman 2002). An additional problem is the occurrence of stacked genes, resulting from crossing between different transgenic events. For the purpose of the unequivocal identification of GMOs, the differentiation between a stacked event, simply called a 'stack', and the individual events from which it originates, is an emerging problem. This in light of the high number of stacked events approved and which method validations are completed (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). DNA quantification methods cannot distinguish between a 100 % GM event containing two genes of two distinct GMOs and a mixture containing 50 % of each of the individual events. Likewise, Van den Eede et al. (2002), Van Duijn et al. (2002), and Wiseman (2002) stated that a sample containing 50 % of a GMO, homozygous for the transgene locus, cannot be distinguished from a 100 % hemizygous line. In an attempt to clarify the GMO content in maize samples imported from the USA in 2005, Akiyama et al. (2008) used the individual kernel analysis system to determine how many stacked GM traits were contained in these samples and which GM trait varieties frequently appeared, the GMO content (percentage) on a kernel basis, and the varieties of the GM kernels in the non-identity preserved (IP) maize samples.

Availability of the GMO CRMs

The applicant for a new GMO in the EU has to provide accessibility to reference materials. In practice, this means that information on where the reference material can be purchased is given by the applicant. Many CRMs for GMO detection can be obtained from the IRMM (http://irmm.jrc.ec.europa.eu/reference_materials_catalogue/Pages/index.aspx). CRMs are certified for the content of the individual GMO. Materials are prepared by quantitative mixing of powder from non-GMO and powder from GMO, produced from ground seeds by a dry-mixing technique. CRMs with different mass fractions are available. Some of these CRMs are also certified for the DNA copy number

ratio. The uncertainty of the GMO content in CRMs is stated and certificates issued. The IRMM also provides a plasmid that is certified for the ratio between DNA fragments of the 5′-end MON-00810-6 transgene–host plant junction sequence and the high mobility group gene A (*hmgA*) (ERM-AD413), with the intention of using this plasmd for the construction of a calibration curve for the quantification of MON810 maize. The American Oil Chemists' Society (AOCS) also produces certified reference materials, available as powder (prepared from 100 % GMO seeds) or leaf tissue DNA, but also as seeds in the case of canola (https://secure.aocs.org/crm/index.cfm). Some reference materials of non-GMO counterparts are also available. The Nippon gene (http://www.nippongene.com/index/english/e_index.htm) is used to produce plasmid reference material and GMO detection kits. The EU Reference Laboratory for GM Food and Feed (EU-RL GMFF), in accordance with the duties and tasks established in the annex to Regulation (EC) No. 1829/2003 (2003) to further validation exercises and the publication of the corresponding reports, distributes the appropriate control samples to National Reference Laboratories that have expressed interest. Žel et al. (2008) showed that these samples are intended to be used for control purposes, while the GMOs are still under the authorization process and no CRM is yet available.

Concluding remarks

The quantification and interpretation of threshold percentages for GMOs are topics that have received particular attention in this review. The units of measurement and expression of GMO contents are closely linked to the type of calibrators used for quantification at the analytical level. In particular, the unit of the used quantitative standards should be consistent with the unit of expression. The determination of GMO content is estimated by means of RT-PCR. The level of GM is calculated by the ratio of the transgene copy number to an endogene copy number. This relative quantification is possible by using standard curves established with CRM. As already mentioned, the production of CRMs is time-consuming, and CRMs are not available for all GM events in Europe. The aim is to used plasmids containing a GMO insert as calibrants for qPCR. Double-target plasmids are a potential replacement candidate for the CRM as pure calibrator molecules for the relative quantification of traces of GMOs in food/feedstuffs. However, due to the large number of GMO events in different species to be authorized worldwide, high-throughput methods that can be used in routine laboratory analysis are needed for the production and validation of double-target plasmids, as well as for their commercialization.

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