SPECIAL GUEST EDITOR SECTION

State of the Art and Limitations of Quantitative Polymerase Chain Reaction

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Consequential to the implementation of European Commission (EC) Regulation 1139/98, EC Regulation 49/2000, and EC Regulation 50/2000 has been the need to measure accurately the levels of the genetically modified (GM) species Roundup Ready Soya and Bt 176 Maize that are present in food. Analytical methods to detect and quantitate these transgenic species have received much attention particularly with respect to the deminimus threshold of 1% for their presence in materials derived from non-GM identity-preserved (IP) supplies. The relative advantages and limitations of threshold analysis by double-competitive polymerase chain reaction (PCR) and quantitative real-time PCR are discussed in their application to the quantitative analysis of processed foods. Consideration is also given to other factors involved in the analyses that affect the performance of quantitative procedures, and to the many uncertainties involved in the precision of a reported analytical result.

European Commission (EC) Regulation 1139/98 (1) required that all food containing ingredients produced from the transgenic cultivars Roundup Ready® Soya (Monsanto) and 'Maximizer' Maize 176 (Novartis) be labeled in all instances where the transgenic protein or the DNA that encodes for the genetic modification (transgene) is present in the food. Since this initial regulation on labeling came into force, it subsequently has been amended to provide for a deminimus threshold of 1% to be set for all such ingredients derived from nongenetically modified (GM), identity-preserved (IP) supplies (EC Regulation 49/2000; 2). These regulations have been extended further to cover additives and flavorings, but no thresholds were introduced for these food components (EC Regulation 50/2000; 3). Consequential to the declaration of a deminimus threshold of 1% was the need to progress from a qualitative detection of the transgenic species, by using an appropriately validated screening system to more complex quantitative procedures. Following the qualitative detection of a genetically modified organism (GMO), manufacturers would be required to label the product, or, in the case of IP sources, determine whether the amount of the GMO(s) present was above or below the 1% threshold. The implication is that the method used to quantitate the level of GMO should be as accurate as possible. The effects of the regulations are such that all aspects of the food chain require detailed analyses involving a large number of food matrixes.

The scientific community has risen to the significant challenges involved and has addressed this difficult problem by using a number of approaches. We have already had comprehensive overviews of how the presence of transgenic protein can be detected and quantitated, together with the limitations of the methods. With regard to using DNA for detection of GMOs, qualitative detection methods commonly use the flanking sequences of the Cauliflower Mosaic virus promoter (Ca Mv 35S) and the NOS terminator from *Agrobacterium tumefaciens* and have been the subject of many discussions. However, a major problem with these detection systems is sensitivity. The Swiss interlaboratory study (4, 5) showed that the sensitivity of 35S detection system varied between laboratories by up to 20-fold. In addition, the detection of the NOS terminator sequence is less sensitive than that of the 35S promoter sequence. The 2 detection systems have also been the subject of a Euopean Union (EU) interlaboratory ring trial (6). Despite increasing experience with these analytical methods, uncertainty still exists, and it is hoped that by moving to quantitative procedures the interlaboratory differences will be minimized.

At present we have no practical methods of analysis that can directly detect and quantitate the very low levels of transgenic DNA found in typical food materials. Radio or fluorescently labeled DNA hybridization techniques can be used to locate specific DNA sequences directly, but their uses and applicability in food matrixes are still to be demonstrated, especially quantitatively. Hence, all DNA targets require amplification to facilitate detection and eventual quantitation. However, although amplification is necessary, it introduces further limitations to the analysis.

The quantitation of DNA targets is a complex process and is the final step in a series of crucial procedures, each of which is equally important to the final analysis. It is imperative to introduce appropriate sampling and DNA extraction procedures for the sample in question. Because these matters have already been discussed at the workshop, this study concentrates on the quantitative analysis of the DNA obtained, particularly

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state-of-the-art methods for quantitating the presence of transgenic DNA sequences, problems encountered, and limitations and uncertainties of the methods. It is hoped that through a consideration of these factors a more general understanding will be reached, enabling an appropriate interpretation to be transmitted to those involved in applying the results of the analysis.

Methods to Detect and Quantitate the Presence of Transgenic DNA

Competitive PCR

(**a**) *General principle.*—The application of competitive polymerase chain reaction (PCR; 7) to the detection of transgenic species within the food chain has recently been reviewed (8). Target DNA is co-amplified in the same PCR reaction as an internal standard. A known amount of the internal standard is added to each reaction. This DNA is specific for each modification and is presynthesized and prepared usually in a cloned plasmid. The plasmid is normally arranged to contain the same modified DNA sequence present in the target but modified to produce a deletion or addition of 20–25 bp in a part of the DNA that is not involved in primer binding. Hence, the same primers will amplify both the DNA sequence present in the specific GMO and in the internal standard. The amplicon produced from the internal standard is 20–25 bp smaller than that produced from the specific target, making differentiation possible by electrophoresis. The internal standard DNA acts as a competitor to the target sequence during PCR, and equivalence is reached when both are initially present at the same concentration, producing the same amount of each amplicon (9). This form of PCR involves the effective titration of a set amount of internal standard with different amounts of target DNA. The relative amounts of each amplicon are usually assessed by amplicon intensity, either by staining, followed by densitometry, or by image-processing software.

(**b**) *Single-competitive PCR.*—A single pair of amplicons is produced (i.e., to the specific target and its competitor). This method should only be considered semiquantitative, as any degradation of the DNA that may have occurred in the sample is not taken into account in the analysis. It is therefore more applicable to raw materials and unprocessed food.

Use of a calibrated internal standard (8) has assisted in making the method more robust and has decreased the occurrence of false negatives as a result of PCR inhibition. It is important to estimate the concentration of DNA used in these forms of analysis, which is usually done spectrophotometrically. A major limitation is that the spectrophotometric estimation of the DNA concentration cannot account for the degree of fragmentation or modification. Hence, the number of copies of amplifiable DNA varies, and the result varies accordingly. Hübner et al. (8) demonstrated that if the appropriate calibration of internal standard DNA is performed, a good correlation ($r^2 = 0.995$) can be obtained and that such calibration is a prerequisite for quantitation. However, the subsequent interlaboratory ring trials showed that reproducibility and precision are unsatisfactory, although these may be a consequence of the samples lacking homogeneity. Threshold analyses can be performed by making the point of equivalence between the 2 amplicons represent a GMO content of 1%; this is achieved by adjusting the amount of internal standard added to each reaction.

(**c**) *Double-competitive PCR.*—This more-powerful technique is a 2-stage process and takes into account both the fragmentation and amplifiability of the DNA. Its use in detecting transgenic material in foods has recently been described (10). An initial examination is made of the amount of amplifiable species-specific DNA present in the sample. Soya content is usually assessed by using the Lectin Lel gene. The sample DNA extract to be analyzed is then diluted accordingly to match the concentration of the Soya lectin competitor. A second set of competitive PCR reactions is then performed to detect specifically the modification in question. It is usual to use the same amount of target DNA but to adjust the internal standard DNA concentration appropriately to an equivalent of 1%. In practice, the PCR components are often premixed for rapid assessments.

Advantages of Double-Competitive PCR

With a threshold method established within a laboratory, a large number of samples can be assessed quickly for analysis of degraded/modified DNA. The method is also advantageous because of the relatively low-cost equipment used.

Limitations of Double-Competitive PCR

Although this method addresses many of the problems associated with the analysis of processed food products, it can only give information relative to the predefined threshold and cannot be applied to give an exact transgene:species ratio. The method described assumes also that amplicons differing by 20–25 bp will be amplified with the same kinetics and efficiency and, in general, DNA fragments >100 bp are required. A number of problems are associated with the staining, densitometry, and imaging aspects of amplicon measurement.

One major problem is that heteroduplex formation between the target and the internal standard alters the ratios of the target and internal standard amplicons produced during the reaction. Heteroduplex formation also complicates the subsequent electrophoretic analysis of the products because of their similar electrophoretic properties.

Real-Time PCR

There are a variety of real-time PCR methods, with a range of different chemistries and instrumentation. The aim, however, remains the same: to follow the PCR reaction and the production of specific amplicons cycle by cycle. This requirement negates the major drawback of gel-based systems, which is to produce sufficient amplicon for visualization by staining an electrophoretic gel. It is normal to perform over 35 cycles of PCR, by which time the exponential phase of the reaction has been inhibited due to end-product inhibition and other complex kinetic effects. This renders the reaction incapable of quantitation and fails to differentiate template DNA concen-

trations differing by a 1000-fold. The ability to follow the reaction in real-time overcomes this particular problem.

Taqman™ (ABI 7700 - Applied Biosystems)

The Taqman™ chemistry exploits the 5′–3′ exonuclease activity (11) of particular *Taq* polymerases to produce a fluorescence signal as a result of the hydrolysis of a fluorescently labeled template-specific oligonucleotide included in the PCR (12). The signal generated is proportional to the amount of specific amplicon produced during the PCR and is detected by a sensitive charge-coupled device, which allows the earliest possible differentiation between the baseline and the true signal. At this early stage, the PCR is operating in an exponential manner that has not yet been subjected to inhibitory kinetics. The point at which the fluorescence signal crosses a predetermined value is recorded and referred to as the cycle threshold (Ct). As with competitive PCR, 2 PCR systems are used to obtain a quantitative result: one to detect the specific transgene and the other a plant-specific DNA sequence that can be used for relative quantitation. These are followed by using different fluorescent reporters. Subsequent analysis generates 2 standard curves and allows the initial ratio of the templates in the reaction to be determined (13). To remove all doubts of inhibition affecting results, particularly when low levels of template DNA are involved, a further internal standard can be used. This is normally arranged to be present in the reaction at the detection limit of the system.

Advantages of Real-Time PCR

The small amplicons (about 80 bp) generally used facilitate better kinetics during the PCR and are equally applicable to unprocessed or processed foods and complex food matrixes and allow the relative ratios of individual transgenes to be determined. The inherent chemistry allows unambiguous amplicon identification due to the number of conditions imposed on the reaction. The 96-well format allows increased replication of the sample and reference material, decreasing the analytical uncertainty involved in critical quantitation analyses, and giving a high throughput and automation potential for qualitative procedures.

Limitations of Real-Time PCR

The major disadvantage is that real-time quantitative measurements involve 2 logarithmic detection systems. The errors involved in determining these values are complex and nonsymmetrical but can be considered as the square root of the sum of the squares of the respective uncertainties. Occasionally the fluorescently labeled probe can be subjected to hydrolysis in some food matrixes systems. Many also consider that the instrumentation is expensive and costly to operate.

Molecular Beacons™ (Stratagene, La Jolla, CA)

These fluorescently labeled reporter molecules behave in a similar manner to the Taqman probes. A specific amplicon is synthesised during the cycles of PCR and a fluorescence signal increases proportionately.

Light Cycler™ (Idaho Technology, Inc., Salt Lake City, UT)

SYBR Green I dye binds to the minor groove of the DNA double helix. The unbound dye produces little fluorescence, which is enhanced significantly when it binds to DNA. The resulting fluorescence is measured at the end of the primer elongation stage of PCR. This method lacks amplicon specificity and hence can be used in all assays of this type. It has the added advantage that a melting curve analysis can be performed after PCR, which gives added confidence to the result.

Hybridization Probes

This format is used whenever maximum specificity is required with regard to amplicon identification. Two sequence-specific oligonucleotides labeled with fluorescent dyes are included in the PCR reaction, the first having a fluorescein label, the second an LC Red 640 label. The design of these oligonucleotides is crucial to optimal performance because both must hybridize within 1–5 nucleotides of each other on the DNA template. If no hybridization to the target DNA takes place, the fluorescein-labeled oligonucleotide once excited simply emits green fluorescence at longer wavelength. However, after hybridization and amplicon production, both labeled oligonucleotides hybridize in close proximity to each other. The emitted energy from the fluorescein-labeled oligonucleotide is transferred by FRET (fluorescence resonance energy transfer) to the adjacent LC Red 640-labeled oligonucleotide, which then emits red light at a longer wavelength. This red light is measured and used to follow the PCR in real-time and is proportional to amplicon generation. This detection mechanism works only when all the components are hybridized; hence fluorescence measurement occurs at the annealing stage of PCR. When compared with the Taqman chemistry, this detection system works better with slightly larger amplicons and hence may lose some sensitivity when small amplicons are used. In contrast, however, Taqman probes appear to hydrolize to produce a fluorescence signal in some applications when DNA from certain food materials is added to the PCR (unpublished data). This presumably occurs as a result of the co-extraction of other materials. The hybridization probes described do not suffer from this problem, but, if degradation occurs, the necessary fluorescence could be destroyed or reduced, affecting the detection limit of the assay.

Factors Affecting Quantitative Procedures

Many laboratories use resin-based DNA isolation and cleanup procedures. The binding efficiency of many of these proprietary materials may differ significantly, particularly with respect to size of the DNA fragments bound. Much more research is required into this area, in particular the subsequent effects on quantitation of DNA targets where severe degradation has occurred. It is also generally assumed that the specific transgene and plant-specific templates degraded equally in all instances and that the amplicons produced during PCR are about the same size. One of the most important criteria in these

analyses is that sufficient DNA is added to each reaction to ensure that an appropriate number of copies of both the specific DNA target and the species-specific endogenous target are present. If this cannot be achieved, quantitative problems will arise. It can be calculated, and is generally accepted, that to achieve a reliable quantitation of the Monsanto Roundup Ready Soya about 10 000 copies of the amplifiable Soya genomic DNA (about 50 ng) must be included in the PCR. This encourages extraction of good quality DNA from the sample, which in turn enables addition of larger amounts of template to the PCR without inhibitory effects.

General Assessment of Uncertainty (Uncertainty Budget)

Abbreviations

Ct—The point, measured in PCR cycles, when a statistically significant increase in fluorescence is first detected.

ΔC_t—The numerical difference between 2 Ct values.

$\overline{C}_{t(fam)}$—The unknown mean of several Ct measurements [probe fluorescently labeled with 6-carboxy-fluorescein (FAM)].

$\overline{C}_{t(vic)}$—The unknown mean of several Ct measurements (probe fluorescently labeled with VIC™).

When reporting results of analyses, one must give meaningful estimates of the uncertainties associated with a particular analytical result. While examining a method, it is important that the precision of the reported result is considered rather than simply taking the precision of the measured values (Ct s) used to generate the results. The coefficient of variation (CV) of a ΔCt is typically 1.5–2%. However, after the reported level of GM material present in the test material is calculated, the value of the CV will invariably be 20–25%. This increase occurs because all of the uncertainties involved in the measurements must be considered, and invariably many aspects must be taken into account, some of which will have a larger effect on the overall uncertainty than others, and some may be so small that they can be effectively ignored for all practical purposes (14). We are obliged to use a logarithmic amplification reaction in our quantitation, and real-time quantitative measurements involve 2 such reactions to define ΔCt or to ascertain relative copy number. These are only 2 of the important measurements within the assessment of the overall uncertainty. These values are complex and nonsymmetrical but can be considered as the square root of the sum of the squares of the respective uncertainties. Using the ΔCt method and assuming that 6 replicate analyses are performed, the unknown means $\overline{C}_{t(fam)}$ and $\overline{C}_{t(vic)}$ are calculated so that the respective standard uncertainties (u) are

$$u_{fam} = \left(\frac{{}^{s}C_{t(fam)}}{\sqrt{6}} \right) \text{and } u_{vic} = \left(\frac{{}^{s}C_{t(vic)}}{\sqrt{6}} \right)$$

where s = standard deviation

Because

$$\Delta C_t = \overline{C}_{t(fam)} - \overline{C}_{t(vic)}$$

The standard uncertainty for ΔC_t is given by

$$u_{\Delta C_t} = \sqrt{\left(u_{fam}\right)^2 + \left(u_{vic}\right)^2}$$

The uncertainty in the position of the calibration curve changes as the ΔC_t changes. We have examined the analysis of a large number of calibration curves and suggest a reasonable estimate for the variation in the Y direction at any point along the curve to be 0.1 ΔC_t.

The combined uncertainty (u_C) is then given by

$$u_C = \sqrt{u_{\Delta C_t}{}^2 + \left(0.1\Delta C_t\right)^2}$$

The expanded uncertainty by using a coverage factor $k = 2$ to give an approximate 95% confidence interval is given by

$$U = ku_C \quad = 2u_C$$

The lower 95% confidence limit, the reported % GMO, and the upper 95% confidence limit are read from the calibration curve at 3 points: $\Delta C_t - 2u_C$ and $\Delta C_t + 2u_C$, respectively. Also, our experience suggests that a large source of uncertainty is in the calibration curve itself. Our standard uncertainty estimate of 0.1 ΔC_t is based on a large number of calibration curves obtained over an extended period of time. However, this is likely to be an over-estimate of the true within-day variation for the calibration.

Other sources of uncertainty not considered in the above budget but presently assumed to be negligible are the purity and accuracy of the standards used in the calibration, the errors in constructing and reading from the calibration curve, the differences between detection instruments, and differences in extraction procedures for DNA. Once uncertainties have been estimated for these aspects of the analysis, they should be added to those already known to provide a better overall estimate of the uncertainty.

Future Considerations

As the number of transgenic species increases, it will soon become essential to have access to individual transgene–plant specific target sequences. This situation is already demonstrated by the use of the Ca Mv 35S promoter sequence. Detection of this promoter alone cannot be quantitative because of its presence in many of the current transgenic species. Other important aspects will be an improved knowledge of number of individual transgenes (i.e., gene stacking) present in each GMO and the availability of appropriate reference materials for all approved and nonapproved releases. It is important to consider future developments in this area, and if transgenes are placed within the chloroplast, quantitation will inevitably be either exceptionally difficult or perhaps impossible. As for the development of new detection methods, DNA arrays

clearly have great potential as screening devices for a large range of transgenic events, but can they be used for quantitation? As our experience grows with the analysis of DNA by capillary electrophoresis, mass spectrometry, and spectroscopy, their roles in quantitative analysis post-PCR should be actively explored. From a theoretical standpoint the detection of transgenes by sequence-based techniques rather than amplification-based methods should be encouraged to avoid the difficulties associated with PCR efficiency and inhibition.

Conclusions

Given the increasing number of approved transgenic crops, more detailed and event-specific information is required to effect quantitation of particular transgenes. It is essential that individuals determine which validated or accredited method is appropriate for their particular analysis and to calculate the uncertainties associated with the analysis of the sample. Those commissioning the analysis do not generally understand the uncertainties involved with the quantitative methods. Hence, it is important to convey the limits of the uncertainty to those using the information without overstating the accuracy.

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