CO-EXTRA

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

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PP Restricted to other programme participants (including the Commission Services)	
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 an approach for quantification of the presence of genetically modified (GM) DNA in samples with low absolute GM content. The approach has been developed in workpackage 5 (WP5) of the Co-Extra project, as a separate task T5.5 “Practical ways to improve limit of quantification for PCR methods”.

The nature of the deliverable is specified as “public report”, and the present report is composed of two parts. Part 1 is a description of the basic nature of the developed approach, its potential benefits and drawbacks, its potential applications and limitations, and proposals to further work and validation of the approach. The other part is a more detailed report on the experiments carried out, the results and interpretations, actual laboratory protocols, etc. Part 2 comprises information that is submitted for publication in a peer review journal with open access, and to avoid breaching copyrights and interfere with the peer review publication process, we chose to refer to the final publication as part 2 of the present deliverable. The manuscript is submitted to an open access journal with the intention that the publication shall be publicly available for free download on the internet. The title of the submitted manuscript is “*Improving hundred-fold the limit of quantification (LOQ) of GMO analyses by qualitative single molecule quantification PCR (SIMQUANT)*” and the authors are Knut G. Berdal, Charlotte Bøydler Andersen, Torstein Tengs and Arne Holst-Jensen.

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2 Part 1 – description of the basic nature of the developed approach, its potential benefits and drawbacks, its potential applications and limitations, and proposals to further work and validation of the approach

2.1 Background

The most common approach to quantify the GM content of agriculturally derived products is by application of real-time quantitative PCR technology. Before real-time PCR methods and equipment became widely available, it was also common to apply competitive PCR. These alternatives (reviewed in Holst-Jensen et al., 2003), however, find limited application when the absolute quantity of the target sequence is low. For real-time PCR, the absolute limit of quantitation (LOQ_{abs}) is usually reported to be between 40 and 100 target sequence copies (Berdal & Holst-Jensen, 2001; Holst-Jensen et al., 2003), due to stochastic distribution of targets in separate subvolumes/reactions. A similar LOQ_{abs} applies to competitive PCR. 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, but also to blending with various ingredients. Consequently, the number of PFU in a template for PCR may be well below the LOQ_{abs} and therefore can not be reliably analysed by application of real-time PCR or competitive PCR. Unfortunately, low PFU does not necessarily imply low relative GM content. For example, in soybean lecithin there may only be 100 PFU of the soybean reference gene present in a DNA extract. If this comes from a 100% GM soybean, then there may also be approximately 100 PFU of the GM sequence present in the same DNA extract. When encountering lower relative GMO content samples, the GM sequence PFUs would be even lower, and with a GMO content below 40%, the GM concentration would be below the theoretical absolute limit of quantification (40 PFU) and yet it would be much above most applied labeling thresholds. These numbers can not be reliably estimated with real-time or competitive PCR due to their inherent LOQ_{abs}. However, for the stakeholders that need to consider the product in relation to current GM legislation and trade contracts, it is not satisfactory to be unable to determine the actual GM content of the product.
The presently described approach was therefore developed as an alternative to real-time and competitive PCR to be able to provide an analytically based GM quantity estimate, with acceptable reliability, from products with absolute GM content below the LOQ_{abs} of real-time and competitive PCR.

2.2 Basic nature of the developed approach

Qualitative PCR is less affected by PCR inhibition than quantitative PCR, because the former only provides a yes/no answer to the question “is the target sequence present in the template for this PCR or not?”. While quantitative PCR estimates the target sequence copy number (PFU) in a specific volume of template DNA, the alternative approach developed here provides quantitative data by determining the presence:absence ratio of standardised volumes and binomial distribution statistics. The approach is closely related to the microbiological most probable number (MPN) approach, and MPN spreadsheets to estimate quantities can also be used to estimate quantities with the presently described approach.
The approach described here is called “qualitative single molecule quantification PCR, or SIMQUANT”. The idea is to divide the original template DNA sample into a subset of several smaller volumes, ideally containing on average 1 PFU of the target sequence per subvolume.

Each subvolume is then subject to a qualitative PCR, and the actual ratio of positives to negatives is then used to estimate the true average PFU content in the subvolumes.

The presence of ≥ 1 PFU in a subvolume shall by definition produce a positive result in the qualitative PCR. The final relative GM content of the product in question can be estimated by taking the true average PFU contents in the subvolumes for the GM target and reference target sequence respectively, adjust for any dilution factors that may have been applied before division into subvolumes, and compare these numbers directly.

2.3 Potential benefits and drawbacks, application and limitations

The described approach can potentially be used to produce analytically based GM content estimates for products that can not be reliably analysed using real-time or competitive PCR. It also does not require availability of advanced real-time PCR thermal cyclers or software, and may therefore be an attractive alternative to permit DNA based GM quantitation for laboratories without access to such technology. However, the approach is more labour intensive because it requires that each sample is analysed by multiple PCR reactions. It may therefore be less cost effective than e.g. real-time PCR where labour costs are high. Furthermore, because the approach may involve post-PCR handling of amplified DNA, there is also a considerably higher risk of carry over contamination, unless the qualitative PCR reactions are performed and analysed in closed systems. Finally, the reliability of the GM quantity estimates depend on appropriate dilution and distribution of the template DNA; as the average PFU content of subvolumes deviates more from 1, the quantity estimate uncertainty increases. And, qualitative PCR is of course affected severely by more or less complete PCR inhibition. Consequently, a PCR inhibition test may be required to exclude the possibility that negative results stem from complete PCR inhibition.
Examples of products and applications include highly processed food ingredients such as liquid soya lecithin, blended products such as animal feeds, and assessment of copynumbers and copy number uncertainty in quality control materials (reference materials without stated uncertainty value; Emons, 2006), prior to their use as calibrants in real-time PCR. We have successfully tested the approach on samples where we have previously been unable to produce reliable quantity estimates with real-time PCR, including proficiency test samples. However, in the absence of any template DNA, or if the DNA is not amplifiable, the SIMQUANT approach can not be used.

2.4 Proposals to further work and validation of the approach

There are many agriculturally derived products where international scientific consensus indicates that it is not possible to produce reliable DNA based analytical quantity estimates. Testing the SIMQUANT approach on DNA from such matrices should be done to assess if this consensus is no longer valid. Furthermore, alternative DNA extraction protocols may find application in combination with SIMQUANT, e.g. because SIMQUANT is less sensitive to PCR inhibitors than real-time PCR. This may potentially allow for the use of faster and/or cheaper DNA extraction methods. Testing of the SIMQUANT approach in other laboratories need to be done, in order to fully validate the approach. We therefore propose to transfer the SIMQUANT approach to workpackage 4 of the Co-Extra project, to consider it for further formal validation. BART (bioluminescent assay for real-time), a reporter system developed by another partner of CoExtra Lumora, will also be evaluated for the possibility to reduce labour intensiveness, possibilities of cross-contamination and costs associated to SIMQUANT.

2.5 Final remarks

The first part of the developmental work was performed in the EU funded project "Reliable, standardised, specific, quantitative detection of genetically modified food" (QPCRGMOFOOD; QLK1-1999-01301). We also acknowledge the Norwegian Research Council for financial support. Finally, the SIMQUANT approach has already found practical application for assessment of target copy number determination for quality control materials used e.g. in research performed in workpackage 6, task 6.6. of the Co-Extra project.

2.6 References

- Berdal KG, Holst-Jensen A: Roundup Ready ® soybean event-specific real-time quantitative PCR assay and estimation of the practical detection and quantification limits in GMO analyses. *Eur Food Res Technol* 2001, 213: 432-438.
- Holst-Jensen A, Berdal KG: The modular analytical procedure and validation approach and the units of measurement for genetically modified materials in foods and feeds. *J AOAC Int* 2004, 87: 927-936.
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- Emons, H. (2006). The 'RM family' – identification of all of its members. Accred. Qual. Assur. 10: 690-691.

Part 2 – the peer review publication with details on protocols, experiments carried out, results, etc.

The submitted manuscript is made available for internal evaluation by the project coordination team, but not for external audience due to copyrights.

A link to the internet publication will be provided here when the final publication is available. It is our goal that it is published in an open access journal, to allow all interested parties to freely download the publication from the internet.