

**Final report of workpackage 1:
“Identification of the domain of application and matrix-limitations for a standard DNA
extraction protocol”**

Project title:
**“Reliable, standardised, specific, quantitative detection of
genetically modified food”**

Acronym: Qpcrgmofood

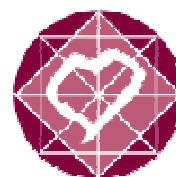


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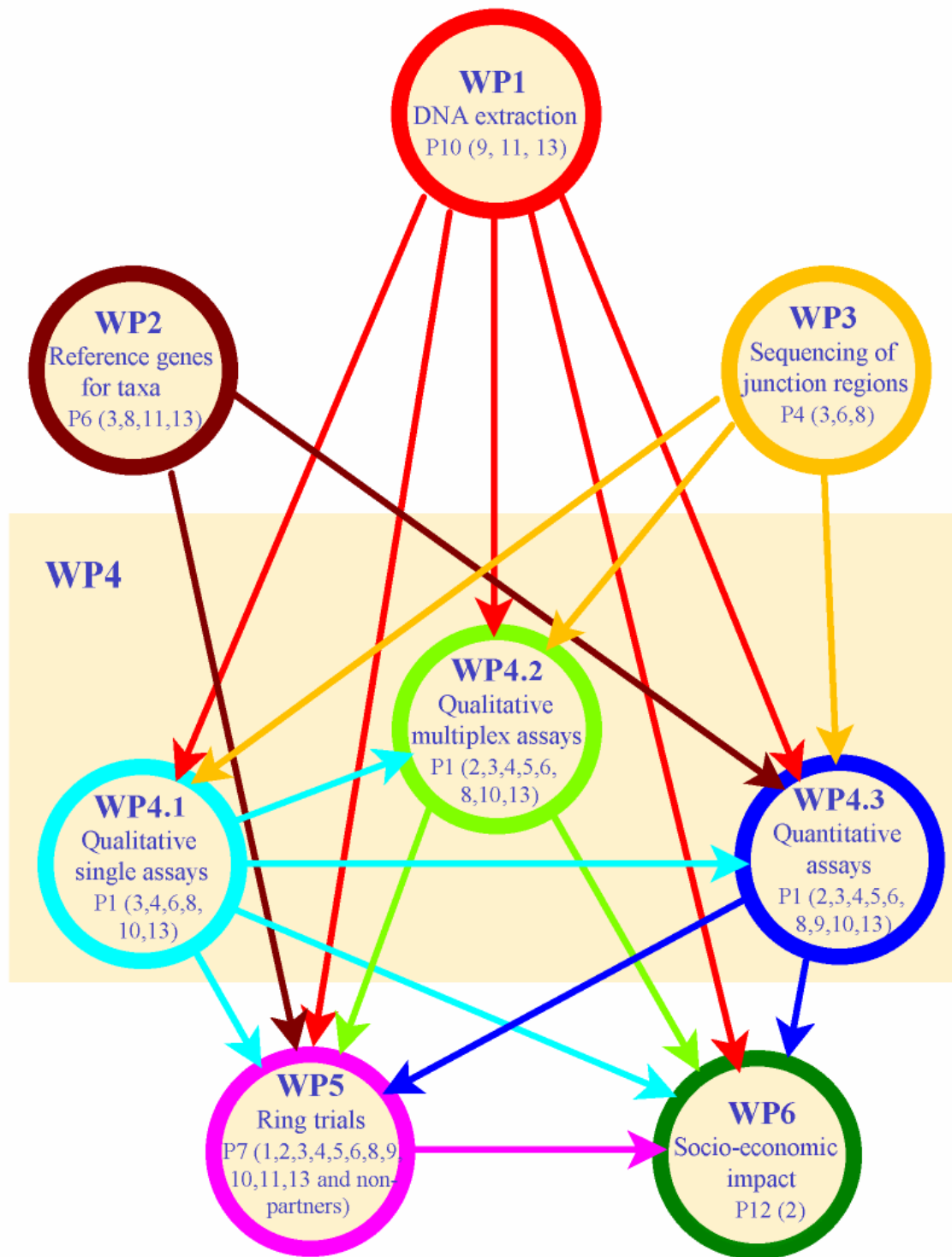
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DELIVERABLE NUMBER 1

Content of this report:

Outline of the Qpcrgmofood project, links between workpackages and partners	3
Summary of the work and results of workpackage 1 (WP1)	4
Overview of partners, objectives and deliverables of WP1	6
Overview of methods developed within WP1	7
Overview of validation status for methods from WP1	8
Other contributions within the responsibilities of WP1	8
Dissemination activities from WP1	9



Outline of project structure, showing links between workpackages and partners

Summary:

Workpackage 1 of the project was responsible for identification of the domain of application and matrix-limitations for a standard DNA extraction protocol

This could be broken up into the following subtasks:

1. Identify the scope and the matrix limitations of a three major types of protocols: CTAB-based, SDS-based (G2) and guanidine-HCl-based DNA extraction protocols.
2. Examine the effect of adding enzymes during the lysis phase.
3. Examine the effect of adding a purification step to the protocols.
4. Modify the protocols or try to develop a specific protocol for the fatty products.
5. Transfer at least one of the protocols to WP5 for validation and forward the validated protocol(s) to CEN for standardisation.

The matrices that have been tested include only soybean and maize (corn) derived products:

Soybean: flour (DNA rich), liquid lecithin (DNA poor, fat rich), solid lecithin (DNA poor), textured protein (DNA rich) and crude oil (DNA poor, fat rich) were tested.

Maize (corn): sweet corn flour (DNA rich), native starch (DNA rich), modified starch (DNA poor), dehydrated glucose syrup (DNA poor), tortilla chips (highly processed, DNA rich but of low quality) and sweet biscuits (highly processed, DNA rich but of low quality) were tested.

Enzyme treatments considered and evaluated:

The following four enzymes and their effects on various matrices were tested: RNase A, Proteinase K, α -amylase and lipase. RNase A generally improved the quality of the extracted DNA, in particular when DNA and protein rich matrices were tested. Proteinase K generally improved the yield of extracted DNA, in particular when DNA and protein rich matrices were tested. No obvious effect of α -amylase alone was observed, although a combination of RNase A, proteinase K and α -amylase improved the total yield and purity more than each enzyme alone when DNA and protein rich matrices were tested. Lipase was observed to have some effect on extraction of DNA from crude oil.

Purification treatments considered and evaluated:

Chloroform extraction, silica binding (QIAEXII or QIAquick), anion exchange resin columns (DEAE) were tested. Although chloroform extraction generally improved the purity of the extracted DNA significantly, and removed soluble PCR inhibitors, this chemical due to its toxicity, is considered harmful. It would therefore be desired to find protocols that do not depend on purification with chloroform. Silica binding and anion exchange resin columns performed more or less equally well. They also improved the purity, but considerably less efficient than chloroform extraction in particular for protein rich matrices. The combination of chloroform extraction and silica binding gave the best results. For matrices with low DNA and protein content, purification is often not required, but will still be recommended on the basis of a cost/benefit evaluation. Presence of chloroform, silica or DEAE in the final extract will have a very clear negative effect on the amplifiability of the extracted DNA.

Approaches for evaluation of extracted DNA:

Two approaches were employed: 1) Inspection of DNA by gel electrophoresis (conventional approach). Determines approximate yield, purity (presence/absence of RNA, polysaccharides and protein), and range of fragment sizes present in the extract. 2) PCR amplifiability with and without PCR inhibition test (for soluble inhibitors, 100 copies of a plasmid was used to spike, and inhibition was determined by quantitative real-time PCR). This test was developed

in the project. The effect of some chemicals (EDTA, NaCl) and contaminants (hydrolysed caseine and various types of DNA) on PCR amplifiability was tested using the PCR inhibition test.

Conclusions:

In general, only DNA rich matrices gave yields sufficiently high to justify validation of the method in collaborative trials (> 10 ng/5 μ l extract), although other matrices also gave reproducible yields (e.g. 1-2 ng/5 μ l extract) when the methods were transferred between laboratories in a prevalidation study. Consequently, it may be possible to validate methods also for DNA poor matrices. However, if DNA is meant to be used for quantitative PCR using e.g. real-time PCR, then the absolute limits of detection and quantitation of the PCR methods (see WP4) may restrict the use of the extracted DNA or require pooling of DNA extracts from multiple extractions from the same sample.

The choice of extraction buffer has significant implications for the choice of subsequent purification steps. This is described in more detail with each matrix specific extraction method, but to give one example, SDS buffer can not be combined with DEAE purification columns.

Protocols tested on sweet maize flour and maize starch were transferred to WP5 for validation on the basis of results from prevalidation (transfer laboratories within WP1 obtained acceptable results). Protocols for soy flour and soy protein have been modified and recent prevalidation results are presently being evaluated, and hopefully this will lead to the decision that the protocol can be validated in a collaborative trial.

Overview of partners, workplan and deliverables of WP1 as described in contract:

The following partners participated in the work performed in WP1:

- P9 Unilever, Vlaardingen, Netherlands
 P10 Direction Generale de la Concurrence de la Consommation et de la Repression des Fraudes, Strasbourg, France
 P11 Groupe d'Etudes et de Controle des Varietes et des Semences, Guyancourt, France
 P13 Consejo Superior de Investigaciones Cientificas, Barcelona, Spain

The workplan of WP1 in the project proposal and contract listed:

The DNA extraction protocol will be tested for its performance on a wide range of matrices, and minor modifications will be introduced to improve performance where suitable. Performance will be evaluated by inspection of quantity and quality of the extracted DNA. Matrices for which the protocol does not yield satisfactory quantity and/or quality of DNA will be identified, determining the matrix-limitations of the protocol.

Objectives

Identify the domain of application and matrix limitations of the CTAB protocol. Examine the effect of adding enzymes such as plant cell wall degrading enzymes (e.g. pectinases, xylanases, cellulases) and other plant polysaccharide degrading enzymes (β amylase...) to the protocol. Examine the effect of adding a purification step to the protocol (silica gel or ion-exchange resin ...) to improve the purity of the extracted DNA.

Modify the protocol or try to develop a specific protocol for the fatty products (specially refined oils) based on an n-hexane treatment before the extraction.

Obtain a protocol for standardisation, e.g. integrated into or compatible with CEN general guidelines. Preferably the protocol should be non-proprietary and user friendly with respect to hazardous compounds and throughput.

Methodology and study materials

The CTAB protocol which we will examine, modify and improve is essentially the procedure previously included in a ring-trial from JRC/Ispra (partner 7). The protocol will be tested on several raw, semi-processed and end-products according to a consensus directory of products still to be defined in discussion with industrials and with the members of the workpackage (e.g. representative starch, derived products). The effect of modifying the protocol by adding various enzymes and purification steps to the protocol will be examined on a matrix-to-matrix basis, and a consensus protocol will be derived from this work. The quality and quantity of DNA extracted will be evaluated by gel electrophoresis, spectrophotometer and gel image analysis.

Overview of deliverables of WP1 listed in project proposal and contract:

<i>Number</i>	<i>Title of deliverable</i>	<i>Nature of deliverable</i>
1	Identification of the domain of application and matrix limitations of a standard DNA extraction protocol	Report (public)

Overview of methods developed:

Target type	Matrices tested	Type of method	Reference¹
DNA and protein rich	Maize flour, Soy flour, soy protein	<p>A CTAB medium scale method with RNase A, proteinase K and α-amylase treatment, chloroform purification and isopropanol precipitation (time to perform method ca. one day) .</p> <p>A simple and quick method based on CTAB buffer incubation followed by a chloroform step and finally a silica column purification (time to perform method ca. 2-3 hours). Compared to above method, less DNA extracted from the soy matrixes, equivalent DNA extracted from sweet maize flour).</p>	<i>P7, P10, P11, P13</i>
DNA poor	Starch	Thermal lysis in presence of CTAB buffer followed by precipitation with isopropanol and purification through a silica column. This method has been validated on native starch.	<i>P7, P11, P13</i>
Fat rich	Lecithin Crude soy oil	<p>Pre-validation of the method showed that, on the tested matrix, the quantity of extracted DNA is insufficient for typical quantitative PCR analysis.</p> <p>Pre-validation of the method showed that, on the tested matrix, the quantity of extracted DNA is insufficient for typical quantitative PCR analysis.</p>	<i>P9, P13</i>
Soluble PCR inhibitors	DNA, EDTA, NaCl, hydrolysed caseine	PCR inhibition test for soluble inhibitors, 100 copies of a plasmid are used to spike a tested solution, and inhibition was determined by quantitative real-time PCR.	<i>P10</i>

¹ Please refer to the original publications for details regarding the methods, since copyrights and other means of protection do not permit details to be published here. A number preceded by a P (both in italics) refer to a partner who has developed but not (yet) published the method. This may be due to confidentiality, need for further improvements of method or because a manuscript is not yet complete.

Overview of validation status for methods from WP1:

Methods have been prevalidated for maize and soy based matrices. On the basis of prevalidation studies, two methods provided by two different laboratories were subjected to validation in collaborative trials; one method for sweet maize flour and one for maize starch. The results seem to support one of the methods, but not the other. A modified version of the latter method has therefore been subjected to another prevalidation study very recently and the results are presently being evaluated before any decision is taken regarding another collaborative trial.

Separation of DNA extraction from PCR (the modular approach) and conducting validation studies accordingly has raised a number of problems (specifically, how to link results with overall method performance). In comparison traditional validation schemes include validation of the combined DNA extraction and PCR steps. Core to this discussion are the following questions: “How do you quantify DNA?”, “How do you assess the quality (size and structural integrity) of DNA?”, and “How do you determine the purity of DNA (absence of inhibitors)?”. Although this is partly discussed in a recently submitted manuscript (Holst-Jensen & Berdal), tools may still have to be further developed for measuring DNA quantity, quality and purity. In the present project the decision was to use validated real-time PCR methods for particular species, e.g. maize (from WP2 and WP5). These real-time PCR methods can be used to answer the fundamental question “Can the extracted DNA be used to quantify GMO with real-time PCR?” If dilution series of the extracted DNA yield Ct-values corresponding to what would be expected from the dilution series, and the shape of the amplification curves indicate absence of inhibitory effects, then the quality and purity of the DNA is considered acceptable. The number of amplifiable copies of the species specific DNA then determines if the extracted DNA quantity allows for quantitation of the GMO by looking at the desired relative limit of detection (LOD) and quantitation (LOQ), and the absolute LOD and LOQ of the GMO detection method.

This discussion has important future prospects for the European Network of GMO Laboratories (ENGL) in the context of the future Community Reference Laboratory (CRL) that is foreseen to be responsible for, among other things, validation of methods under the requirements of the new GM-food and -feed legislation.

A multiauthored paper reporting on the methods and validation study is in preparation for a peer reviewed international scientific journal.

Other contributions within the responsibilities of WP1:

Apart from providing data on methods and effects of various modifications on the extractability and quality of DNA from various matrices, the development of general principles for validation of DNA extraction methods separately from validation of e.g. PCR methods undoubtedly represent a major step forward in relation to discussions about the possibility for validating methods on a modular basis. Modularity will eventually introduce more flexibility and potentially reduce the costs associated with method validation. This is because methods may be combined in the analysis of a laboratory sample, e.g. a separately validated matrix specific DNA extraction method, species specific PCR method, and GMO specific PCR method.

Dissemination activities from WP1:Training, exchange, etc.:

Oral presentation of WP1 results by Carlo Bulkman (P9) to GEMOBILITY project meeting (QLK1-1999-00527), Institut für Mikrobiologie und Genetik, University of Vienna, Austria, March 26th 2001.

Scientific publications from WP1 (peer reviewed journals, books):

In preparation.

Oral and poster presentations from WP1 at scientific conferences, etc.:

Presentations foreseen on the basis of the final results of the validation studies, in the context of ENGL, and by preparation of method annex(es) to CEN/TC 275/WG 11 and ISO/TC 34/WG 7.