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Guidance on the selection and use of DNA extraction methods

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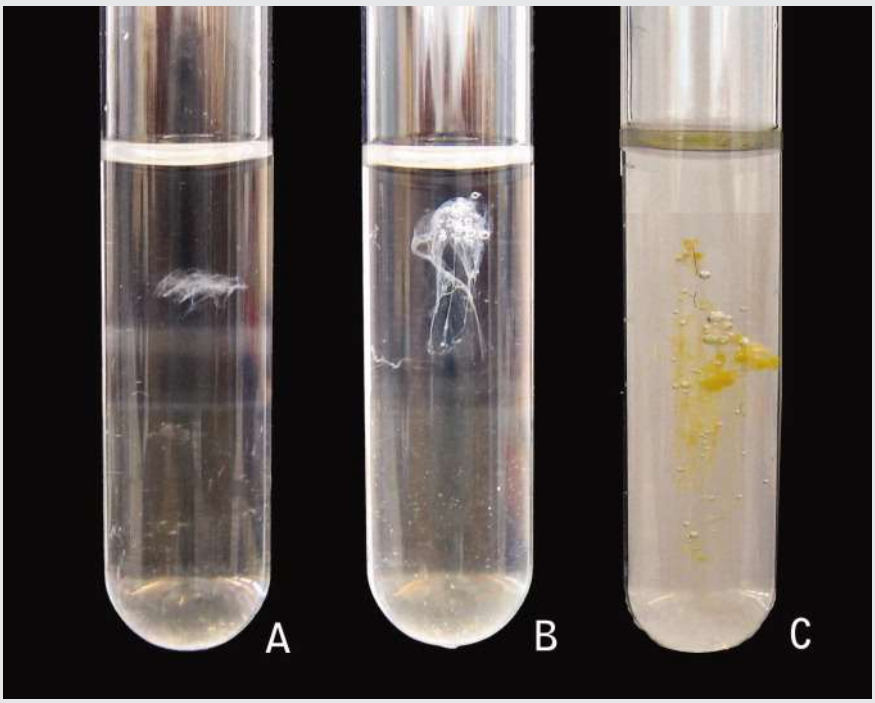

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Contents

Abstract

DNA extraction is at the forefront of further analytical measurements on DNA targets and affects the downstream results. This report from the European Network of GMO Laboratories (ENGL) provides guidance on the selection and use of fit-for-purpose DNA extraction methods. It focusses on DNA extraction in the context of official controls on the presence and content of genetically modified organisms in food and feed. It provides guidance on protocols and selection support systems, validation approaches, assessment of DNA quality parameters and examples of practical solutions derived from collective experiences. There are many variations on the theme of DNA extraction, but there is no single protocol that works adequately across all food and feed matrices. Before using a new method in the laboratory, or in case of modifications to a protocol, validation or verification is needed to show that a chosen method is fit for purpose for use in routine analysis. This guidance is aimed to help the DNA analysis laboratories in fulfilling the standardisation requirements and support their daily operations.

Foreword

The subject of this guidance document is DNA extraction for GMO analysis in the context of official controls according to Regulation (EU) 2017/625, fulfilling the requirements under Regulations (EC) No 1829/2003 regarding the labelling of food and feed, (EC) No 1830/2003 regarding traceability, and (EU) No 619/2011 regulating the presence of traces of certain GMOs in feed.

The scope of this document is to provide guidance on the selection and use of suitable DNA extraction methods and protocols, their validation, and the assessment of DNA quantity and quality. Practical examples of decision support systems for selecting an appropriate DNA extraction protocol are presented. Practical solutions based on experiences are provided for laboratory technicians working daily with DNA extraction protocols and guidelines are described for DNA extraction from particularly challenging types of matrices.

The extraction of DNA from a food, feed or seed sample that is of an appropriate quality and sufficient quantity for further analysis is the first step in the analytical procedure for GMO testing. Many different extraction methods, utilising various principles, have been developed and are used to extract DNA from such samples. In-house developed methods, commercial kits and commercial automated DNA extraction systems are available. Many modifications of the basic approaches are applied for specific samples in different laboratories. Some methods may be more suitable for certain matrices than others, and the quality of the extracted DNA may affect the downstream analytical steps.

In 2017, the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) organised a training workshop on DNA extraction from food and feed and collected information from ENGL members in a questionnaire on this topic. Differences were identified among approaches applied by laboratories to isolate DNA from different matrices. At the same time, incidents were reported where the DNA extraction method applied seemed to affect the final GM quantity measured. It was concluded that further harmonisation in the selection of a DNA extraction method for a particular sample matrix would be welcome and that there was a need for providing guidance on this.

In order to capture the current knowledge and experiences of participants and make it available to a wider audience, the Working Group (WG) on DNA extraction was established. One of the main tasks of this WG was to produce a Guidance Document by giving consideration for inclusion of the following topics:

- Summarising the knowledge on DNA extraction methodologies (including purification, analysis of DNA quantity and quality) reported in the literature incl. international standards, and within ENGL;
- Providing best practices in the selection of DNA extraction methods and DNA quality assessment, and their applications to food, feed, seed and plant matrices;
- Providing practical guidance on in-house validation and verification of DNA extraction methods;
- Grouping sample matrices according to relevant criteria that would affect their DNA extraction requirements, e.g., proximate composition (fat, protein, carbohydrates), or degree of processing;
- Comparing characteristics and limitations of various DNA extraction kits on the market and of the different CTAB DNA extraction approaches used;
- Providing a list of sample types and corresponding options for their DNA extraction method incl. recommended modifications;
- Developing a decision matrix for the selection of DNA extraction methods that are suitable for certain food, feed and seed types;
- Reviewing the acceptance criteria for DNA quality assessment for various analytical downstream steps.

Based on this mandate, the Working Group established five task groups, focusing on:

1. DNA extraction methods, protocols and guidelines;
2. DNA extraction method validation/verification;
3. DNA quality and quantity measurements;
4. Recording practical experiences in a DNA solutions table;
5. Decision support systems for DNA extraction.

The structure of this guidance document reflects the contributions prepared by each of the task groups. Annexes are also included providing examples of DNA extraction for challenging samples or matrices.

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Executive summary

GMO analysis by analytical laboratories relies on the quantity and quality of the DNA extracted from test samples to successfully perform real-time or digital PCR experiments in fulfilment of their duties regarding the monitoring and quantification of GMOs. This is particularly important in the context of official controls according to Regulation (EU) 2017/625, fulfilling the requirements under Regulations (EC) No 1829/2003 regarding the labelling of food and feed, (EC) No 1830/2003 regarding traceability, and (EU) No 619/2011 regulating the presence of traces of certain GMOs in feed.

In order to share experiences, this JRC technical report on 'Guidance on the selection and use of DNA extraction methods' is written to inventory a non-exclusive set of DNA extraction methods and DNA quality/quantity assessment approaches that can benefit GMO analysis laboratories. The report is intended as a source for specific questions or topics that can be consulted in the respective Chapters.

The aim of this technical report (**Chapter 1**) is to provide practical guidance regarding the selection and use of suitable DNA extraction methods and protocols, and their validation, and on the assessment of DNA quantity and quality. Decision support systems for the selection of appropriate DNA extraction protocols are described, focusing on practical examples. Common issues and practical solutions, based on real-life experiences, are described as an aid for laboratory personnel working routinely with DNA extraction protocols. Finally, guidance is provided for DNA extraction from particularly challenging types of matrices.

Chapter 2 of this report reviews the basic features associated with the high diversity of food and feed samples and the most frequently applied DNA extraction methods. Focus is given on DNA as the target of choice since GMO detection and quantification is predominantly performed with DNA-based methods. This chapter reviews existing method protocols and guidelines on DNA extraction from literature.

In **Chapter 3**, the parameters and techniques that can be used to assess the quality and quantity of the extracted DNA are described. Practical case studies are presented to demonstrate how such quality checks can be performed in routine analysis. The chapter particularly discusses challenges with food, feed and seed samples. Depending on the laboratory's individual situation, different approaches can be applied**.**

In **Chapter 4**, approaches are described for the validation of a DNA extraction method before its use for official control purposes. Examples are provided for the interpretation of the criteria that describe the DNA quality and quantity and its suitability for downstream applications such as real-time or digital PCR. This chapter also points to the steps to be taken when a method is proposed as a national or international standard.

Chapter 5 provides examples of decision support systems (DSS) allowing selection of the most suitable DNA extraction method for a given sample matrix. This is based on practical experiences from ENGL members.

For common problems encountered during DNA extraction a number of practical solutions, as well as a trend analysis based on collective ENGL experiences with DNA extraction, are presented in **Chapter 6**.

In addition to the main chapters of this report, several DNA extraction procedures are described in separate annexes for very specific and challenging food matrices.

1 Introduction

DNA extraction is defined as “Separation of the DNA from the other components in a test sample” (ISO 24276:2006/A1:2013). Generally, any DNA-based detection method includes a DNA extraction protocol that is applicable to the matrix of the sample. For each DNA detection or/and quantification method in bio-molecular analysis, at least one extraction method should be available, tested and validated as stipulated by the Codex Committee on Methods of Analysis and Sampling (CCMAS, 2010).

The choice of DNA extraction method should consider the type of matrix and the downstream application(s). It is necessary to closely evaluate the suitability of a method. For instance, in the case of detection of trace amounts of an analyte, the aim can be to detect analytes that may be present (and need to be quantified) in only one copy per thousand copies of a haploid genome.

The essence of extraction is to obtain a sufficient quantity of DNA with suitable quality for subsequent analysis. Quantity refers to an amount of extracted DNA sufficiently high to produce reproducible results when the downstream method is applied (CCMAS, 2010), while quality refers to the length, structural integrity, and physico-chemical purity (ENGL, 2015). Purity refers to being free from impurities and/or contaminants that can inhibit the subsequent enzymatic reactions, e.g., the activity of the DNA polymerase during PCR. In a broader sense of the word, purity may also refer to the degree or extent of degradation. For extraction from intact cells or tissues, the DNA should not be degraded more than is feasible to get the DNA into solution. The DNA integrity depends in many cases on the original state of the DNA in the matrix. DNA extracted from highly processed food and feed samples is generally more sheared and of lower molecular weight and may also include a significant amount of single stranded DNA. For some matrices, a lower extraction efficiency or high degradation may be acceptable. In this case, the report of analysis should include information on the condition of the matrix. In general, it is important that the DNA is extracted in a manner that ensures the representativeness of the DNA in the sample. For example, for a mixture of degraded and non-degraded DNA in a compound sample, the extraction of the intact DNA should not be favoured – although this may be difficult in practice.

The extraction efficiency of the method and the DNA purity estimate should be assessed for the particular matrix (CCMAS, 2010). Once the integrity and purity are assessed, it is decided whether the DNA is suitable for the subsequent steps.

According to the guidelines of the ENGL document 'Definition of minimum performance requirements for analytical methods of GMO testing' (ENGL, 2015), the DNA concentration should be appropriate for the PCR analyses. Structural integrity of DNA fragments means that the average DNA fragment length should be higher than the PCR amplicon size in subsequent analyses. To assess the amplifiability of the DNA an inhibition test may be performed, as described in the ENGL Method Verification Document (Hougs et al., 2017).

As GMO detection and quantification mostly rely on DNA-based methods, Chapter 2 of this report reviews the basic features of the high diversity of various food and feed samples and of the most used DNA extraction methods. Also, future challenges of DNA extraction methods are discussed.

As laboratories have to show that the extraction method used is fit for its intended purpose, a validation or verification procedure is highly recommended. Depending on the laboratory’s individual situation different approaches can be applied. Chapter 3 demonstrates potential approaches which are discussed by means of practical case studies.

Chapter 4 presents the parameters and techniques that can be used to assess the quality and quantity of the extracted DNA. Chapter 5 provides examples for decision support systems allowing selection of the most suitable DNA extraction method for a given sample matrix. For common problems encountered during DNA extraction a number of practical solutions, as well as a trend analysis based on collective ENGL experiences with DNA extraction, are presented in Chapter 6. In addition to the main chapters of this report, a number of DNA extraction procedures are described for very specific challenging food matrices in separate annexes.

2 DNA extraction in function of GMO analysis in food and feed: Review of methods, protocols and guidelines

2.1 Introduction and objective

DNA extraction is a basic and crucial step for DNA-based analysis of GMOs. Steps in DNA analysis may include screening, identification and quantification of one or more GM events by quantitative real-time PCR (qPCR) or digital PCR (dPCR), and sequencing. Extracted DNA has to be of high-quality (as described in Chapter 1) and quantity that is sufficient for downstream applications. Moreover, it should be representative for the DNA composition of the whole sample. A wide range of DNA extraction methods have been used for plant-derived samples, from seeds to highly complex and processed feed and food products. Several outcomes from comparative interlaboratory studies are available. Nevertheless, extensive reviews of suitable methods for various matrices, particularly with respect to GMO analysis and taking into account all factors that are relevant for the downstream analyses, are still missing. Due to the huge diversity of samples and their features, either chemical or physical, there is a need to elucidate and bring new insights on how DNA extraction may influence GM event detection and quantification results. This chapter provides an overview of existing commercialized and/or peer-review published methods for DNA extraction in function of GMO analysis. DNA extraction protocols are described in view of their general principle, but also coupled to the type of matrix/matrices for which they apply.

In eukaryotes, nuclear DNA is localised in the nucleus of the cell. Cell membrane solubilisation and inactivation of intracellular components, although initiated by the mechanical actions, are typically achieved by chemical methods based on the activity of lytic enzymes, chaotropic chemicals and detergents (Querci et al., 2020). After the lysis and the extraction, DNA often needs to be purified further. In order to separate the debris from the solution or the organic from the aqueous phase during the extraction, centrifugation may be helpful. Centrifugation is combined with column chromatography (spin columns) in certain DNA extraction kits. These and other steps for further DNA purification, e.g., precipitation, chromatography or other separation techniques, are also subject of and included in this chapter.

2.2 Laboratory sample preparation

Differences in physical properties of the sample e.g., cell wall composition, cell content chemical composition, particle size distribution in the sample and physical state (solid, liquid, highly viscous etc.) may affect extraction efficiency.

Cell wall disruption/crushing and homogenisation of particle size can be achieved using a physical method with an appropriate device (knife mill, sieve mill, homogeniser). Pre-treatment of particularly acidic, fatty, or starchy samples is also required to aid extraction of DNA and improve efficiency of cell lysis. **Table 1** summarizes a number of homogenisation methods and pre-treatment approaches of food samples prior to DNA extraction with advantages and disadvantages.

Table 1. Homogenisation and pre-treatment of food samples prior to DNA extraction (from Terry et al., 2002)

Procedure	Method	Usage/results	Advantages	Disadvantages
Homogenisation	Stomach blender	Good for flour, protein powder, tofu, soybean sausage	No contamination	Insufficient mixing with soybean and maize
	Mortar/mill	Good for samples with (leaf) particles, e.g. soybean, maize, grist	Good mixing	Risk for contamination
	Stirring/ shaking	Good for liquid samples	Low/No risk for contamination	Liquids only
Pre-treatment	NaOH treatment	Neutralizes acidic samples	Improves extraction from soy sauce, ketchup, fruit juice	
	Hexane treatment	Removes fat from a sample allowing dispersion in aqueous buffer	Improves extraction from fats, oils, fat-containing samples	Additional step in protocol, using toxic substance
	Alpha-amylase treatment	Enzyme hydrolysis of starch avoids gelatinization and DNA capturing	Improves extraction from starch-containing samples	

2.3 DNA extraction and purification methods for food and feed samples

The choice of the most suitable DNA extraction technique should include the following criteria:

- Target nucleic acid(s)
- Source organism(s)
- Starting material (tissue, leaf, seed, processed material, etc.)
- Desired results (yield, purity, purification time required, etc.)
- Downstream application(s) (PCR, cloning, labelling, blotting, qPCR, cDNA synthesis, etc.) (Querci et al., 2020).

Currently, there are many specialized methods, and these can be divided into solution-based or column-based protocols. Most of these protocols have been developed into commercial kits to simplify the DNA extraction processes (Tan & Yiap, 2009).

2.3.1 General considerations for downstream applications

Acceptance criteria applicable to DNA extraction methods are defined in the ENGL document on Definition of minimum performance requirements for analytical methods of GMO testing (ENGL, 2015). Only DNA of certain quality is amplifiable in further analysis using molecular methods. The influence of the extraction method and of sample characteristics/properties on the yield and quality of DNA extracted from different samples has been reviewed (Gryson, 2010; Demeke & Jenkins, 2010) and both have a crucial influence on the results of GMO detection and quantification (Cankar et al., 2006).

The methods are typically used in combination, such that a number of pre-treatments and purification steps may be applied to optimize the extraction procedure, depending on the type of matrix. Different enzymes can e.g., be used in the lysis of matrices. However, the purity and quality of DNA must be balanced with yield (Terry et al., 2002).

The EURL GMFF validates the analytical methods submitted by applicants for authorisation of a new GM event, including the DNA extraction method proposed by the applicant. It is relevant to mention that extraction methods validated by the EURL GMFF are analysed on very simple matrices (ground seeds) and not on other more complicated matrices typical of food or feed materials. Furthermore, the interlaboratory validation of the quantitative event-specific PCR methods is performed on already extracted DNA provided by the applicants. This means that it is only during proficiency tests on complex matrices that the limitations of such validations are seen.

2.3.2 Most commonly used DNA extraction procedures

Generally, successful extraction of pure DNA requires the effective disruption of cells or tissue, the denaturation of nucleoprotein complexes, the inactivation of nucleases and successful removal of contaminants (Doyle & Doyle, 1990).

The most frequently used DNA extraction procedures are based on the following principles:

1) Organic extraction (variations of phenol/chloroform method) or the use of a multistep liquid chemical process. In this conventional, widely used approach, cells are lysed in the presence of a detergent (CTAB: cetyltrimethylammonium bromide; SDS: sodium dodecyl sulfate), and cell debris is usually removed by centrifugation. Then, proteins are denatured/digested using a protease, and precipitated with organic solvents such as phenol and chloroform. The protein precipitate is removed following separation by centrifugation. Purified DNA is usually recovered by precipitation using ethanol or isopropanol. At some point in the process, RNAs are degraded through incubation with RNase. Despite the toxicity of phenol, and residual phenol or chloroform that may affect downstream applications, organic separation is still widely used.
2) Silica-based technologies are widely employed in commercially available kits. DNA adsorbs specifically to silica membranes / beads / particles in the presence of certain salts and at a defined pH. The cellular contaminants are removed by wash steps. DNA is eluted in a low salt buffer or elution buffer. Chaotropic salts are included in the kit buffers to aid in protein denaturation and extraction of DNA. This method can be incorporated in spin columns and microchips, is cost-effective, has a simpler and faster procedure than the organic extraction, and is suitable for automation (Tan & Yiap, 2009).

3) Solid phase extraction methods: magnetic separation technology is based on DNA reversibly binding to a magnetic solid surface, bead and particles that have been coated with a DNA binding agent (Kroken et al., 1997). After DNA binding, beads are separated from other contaminating cellular components, washed, and the purified DNA is eluted using ethanol extraction (Ma et al., 2013). This method is rapid, simple to perform and can be automated. However, it can be more costly than other methodologies.

4) Anion exchange technology DNA extraction by anion exchange chromatography is based on the specific interaction between negatively charged phosphates of the nucleic acid and positively charged surface molecules on the substrate. DNA binds specifically to the substrate in the presence of low salt, contaminants are removed by wash steps using a low or medium salt buffer, and purified DNA is eluted using a high salt buffer (Budelier & Schorr, 2001).

5) Other methods of DNA extraction include salting out, caesium chloride density gradients and Chelex-100 resin (Bio-Rad) (Aljanabi and Martinez, 2021; Miller et al., 1988; Walsh et al., 1991; Garger et al., 1983; De Lamballerie et al., 1992).

2.3.2.1 CTAB-Methods

The CTAB protocol was first developed by Murray and Thompson (1980) and published by Wagner et al. (1987). The method is appropriate for the extraction and purification of DNA from plants and plant-derived foodstuff and is particularly suitable for the elimination of polysaccharides and polyphenolic compounds otherwise affecting the DNA purity and therefore quality. This procedure has been widely applied in molecular genetics of plants and was already tested in validation trials in order to detect GMOs (Querci et al., 2020) (Chapter 3).

In the basic CTAB method (for plant and plant-derived matrices) the lysis step is followed by several purification steps in order to remove contaminants. Chloroform is used to separate the nucleic acids from CTAB and polysaccharide/protein complexes. The final purification of the nucleic acids is conducted by isopropanol precipitation and washing with ethanol at 70% (ISO, 2005).

2.3.2.2 CTAB-based method variants

Several additional variants have been developed to adapt the method to a wide range of raw and processed food matrices (Hotzel and Sachse, 1999; Hupfer et al., 1998; Meyer, 1999; Poms, 2001, 2004; Querci et al., 2020).

Overall, "CTAB" DNA extraction methods are used by approx. 82% of the EU GMO laboratories that took part in the EU Survey on DNA extraction from food and feed in 2017 (Annex III). CTAB-based extraction methods are frequently used on samples derived from plant materials, either fresh fruits, leaves or seeds. However, it is also the method of preference for various food samples (Marmiroli et al., 2008; Smith et al., 2005; Turci et al., 2010). CTAB is nominated by a majority of the labs (% of 2017 EU survey (Annex III) participating labs indicating that they use CTAB per type of matrix) for maize seed (58%), soybeans (55%), oilseed rape seeds (39%), rice seeds (39%), vegetables and fruits (32%), and to a lower degree also for cottonseed (18%), vegetable seeds (18%), potatoes (16%) and sugar beet (13%). For processed matrices, CTAB-based protocols are used for plant-based (45%) and animal-based feed (21%); feed additives (21%); for carbohydrate-rich (45%) as well as fat-rich (42%) and protein-rich (39%) processed foods; for highly processed food such as gluten and lecithin (37%); for chocolate (18%) and honey (13%) (Annex III).

The CTAB-based approach is useful for the DNA extraction and purification in matrices with high content of polysaccharides, as mentioned also by Smith et al. (2005), for potato tubers, and polyphenolic compounds.

According to Mafra et al. (2008), CTAB methods are better for the difficult processed soy-based samples (soybean drinks, desserts, vegetarian foods), than for extraction of DNA from soybean flours, protein isolates or tofu. Also, Hrnčírová et al. (2008) demonstrated that CTAB methods are more suitable for processed soya (e.g., granules) than for raw materials. Slight modifications to the basic CTAB extraction may be applied, such as addition of Proteinase K or other agents to remove impurities more easily from the cells or the DNA extract (Table 2).

The CTAB method was suitable for DNA extraction from honey, but the DNA quantity was affected by the treatment of the samples. Without the use of glass beads for pollen grain disruption, the method allowed amplification of the tRNA-Leu and actin plant-specific genes (Torricelli et al., 2016). Pre-treatment without disruption of pollen or using an ultrasonic bath was not effective enough for sufficient DNA extraction. Pure and highly concentrated DNA can be obtained with CTAB reagents and a combination of solutions with different

salt concentrations and a number of purifications with chloroform treatment. The last treatment with 70% ethanol allows the last purification of the nucleic acid from the remaining salt.

A good compromise between quantity and quality of the DNA extracted from honey is achieved by Torricelli et al. (2016) by modifying and adding a final NucleoSpin gDNA purification step to the combination of pre-treatment and extraction method described in the German guideline (BVL, 2011). This protocol was validated with DNA extracts from honey and pollen and applied to 18 commercial honey samples.

Marmiroli et al. (2008) report on a comparative study between different DNA extraction methods in terms of DNA yield, the presence of inhibitors, required time per extraction, and costs per sample. They concluded that CTAB generally performed best, compared to the PVP method and a list of commercial kits. In a later study however, Žel et al. (2015) state that in terms of costs per sample and depending on the number of samples extracted at the same time, CTAB is more expensive than many commercial DNA extraction kits, as it mainly requires a longer hands-on time. It is very difficult to compare costs of DNA extraction methods in terms of one single parameter. Also, the cost per sample will depend highly on the number of samples extracted simultaneously and thus also on aspects including the degree of automatization of lab equipment, training level of the personnel, etc. Different aspects should thus be considered before making conclusions regarding the most expensive or cheapest DNA extraction method.

In CTAB-based methods, the DNA extraction buffer is usually composed of 2% (w/v) CTAB (Smith et al., 2005) combined with different variants of organic compounds such as β-mercaptoethanol and phenol/chloroform or phenol/chloroform/isoamyl alcohol. Many protocols comprise several steps of purification, which make these methods quite laborious, but facilitate the extraction of large amounts of pure DNA. There are also versions adapted to a kit format (Tables 3 and 4: examples of the most used methods and protocols).

Table 2. Overview of CTAB-based DNA extraction protocol variants

Methods/Extraction buffer	Purification	Matrices	References
CTAB	Chloroform Isopropanol + ethanol 70% wash	Wide range of raw and processed food and feed matrices and seed. Highly processed soybean flour	ISO 21571:2005; ISO 21571:2005/A1:2013; Corbisier et al. (2005)
CTAB +/– Proteinase K, +/–RNase	Chloroform Isopropanol + ethanol 70% wash + Clean Up	Increased solubilisation, digestion, purity and quality in protein rich and processed food products. honey, pollen	Mafra et al. (2008); Marmiroli et al. (2008); Stefanova et al. (2013); Smith et al. (2005); Cankar et al. (2006); Corbisier et al. (2007); Sisea & Pamfil (2007); EURL method CRLVL26/04XP Version 1 (2017); Torricelli et al. (2016)
CTAB +/– Proteinase K, +/– RNase (+ pre-treatment lipase or n-hexane)	Chloroform Isopropanol + ethanol 70% wash	Matrices rich in fat	Demeke & Jenkins (2010); Pirondini et al. (2010)
Higher CTAB concentration/salts + β-mercaptoethanol/ PVP	Ice cold Isopropanol + ethanol 70% wash	Matrices rich in polysaccharides and phenolic compounds	Maltas et al. (2011)
CTAB /*N*-phenacylthiazolium bromide (PTB)	Chloroform/butanol (24:1)	Highly processed foods (mechanical/thermal/chemical treatment)	Di Bernardo et al. (2007)

2.3.2.3 Silica-column-based methods

After CTAB-based protocols for DNA extraction, silica column-based protocols are the second most used group of DNA extraction methods for GMO analysis. The following kits are generally mentioned as being used most frequently in EU labs: Macherey-Nagel NucleoSpin Food (approx. 40%), Qiagen DNeasy Plant Mini (26%), Promega Wizard (21%), Qiagen DNeasy Mericon Food (18%), and Macherey-Nagel NucleoSpin Plant (16%) (Annex III). Most of these methods are commercialized under a kit format (Table 3). Basic silica methods using a generic lysis buffer and are recommended for a wide range of matrices with the exception of fat-rich matrices. After a lysis step, followed by a purification step, contaminants are washed from the resin by

isopropanol, with a final elution step with a low-salt buffer solution. Advantage is avoiding the handling of highly toxic chemicals (21571:2005; ISO 21571:2005/A1:2013).

Overall, DNA extraction kits may be more time-efficient and user-friendly, however not necessarily cheaper. Also, several authors reported that using kits may result in smaller yields of DNA, with variable degree of degradation and highly variable amplifiability of the target DNA by PCR. For instance, The GeneClean II Kit (Q-BIOgene) allows for the amplification of the taxon-specific lectin gene in all soybean-derived matrices (from minimally processed to highly processed foods) but the same was not observed for the element-specific CP4-EPSPS; The Wizard Genomic and the NucleoSpin Plant gave better results on the detection of taxon-specific zein gene in maize-derived matrices (all processed foods) than the GeneClean II kit (Q-BIOgene), which produced some false negatives results (Marmiroli et al., 2008).

Table 3. Basic commercial silica-column DNA extraction kits

Protocol	Link to basic info on protocol and applicability (matrices)	Other matrices (examples)	Reference (other matrices tested)
NucleoSpin Food (Macherey-Nagel)	https://www.mn-net.com/nucleospin-food-mini-kit-for-dna-from-food-740945.50	Tomato derived matrices; meat products	Turci et al. (2010); Sovova et al. (2017)
NucleoSpin Plant (Macherey-Nagel) Wizard DNA Extraction and Cleanup Resin (Promega)	https://www.mn-net.com/nucleospin-plant-ii-mini-kit-for-dna-from-plants-740770.50?c=3895, https://be.promega.com/products/nucleic-acid-extraction/clean-up-and-concentration/wizard-dna-clean-up-system/?catNum=A7280#overview	Maize derived (all processed food); processed soybean foodstuff; honey; feeds highly processed (pelleted feeds, expanded feeds and coarse mixes)	Marmiroli et al. (2008); Mafra et al. (2008); Soares et al. (2015); Jasbeer et al. (2009)
DNeasy Plant Mini (Qiagen)	https://www.qiagen.com/us/search/products/?query=dneasy%20plant%20mini%20kit%20en	Jam; Highly processed soybean flour	Sovova et al. (2017); Corbisier et al. (2005)
DNeasy Mericon Food (Qiagen)	https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-mericon-food-kit/	Processed food products; raw muscle, heat-treated muscle, and homemade meat; gelatin	Coello et al. (2017); Piskata et al. (2019); Shabani et al. (2015)
MP Biomedicals FastDNA Kit, GeneClean Kit (Fisher Scientific, Thermo Fisher Scientific)	https://www.fishersci.com/us/en/browse/90222171/dna-extraction-and-purification?filter=brands_ss%3AMP%20Biomedicals%2C%20Inc	Soy derived (raw and processed)	Marmiroli et al. (2008)
SureFood Prep Advanced Art. No S1053 (R-Biopharm)	https://food.r-biopharm.com/products/surefood-prep-advanced/	Highly processed food and feed products for GMO, allergen or animal identification analysis	PDF manual on https://food.r-biopharm.com/products/surefood-prep-advanced/
FoodProof Sample Preparation Kit III (Biotecon Diagnostics)	https://www.bc-diagnostics.com/products/kits/sample-preparation/foodproof-sample-preparation-kit-iii/	Demanding isolation and purification of DNA from raw materials and foodstuffs of plant or animal origin for GMO, allergen or animal ID analysis by qPCR	PDF manual on https://www.bc-diagnostics.com/products/kits/sample-preparation/foodproof-sample-preparation-kit-iii/

Other silica-based kits:

- GeneSpin (GeneScan (now Eurofins)), Cankar et al. (2006);
- QIAamp DNA Stool Kit (Qiagen), Marmiroli (2008);
- UltraClean Plant DNA Kit (Mo-Bio), Smith et al. (2005);
- Hi-Pure PCR Template Preparation Kit (Roche), Smith et al. (2005);
- DNA Isolation Kit for Cells and Tissues (Roche), Smith et al. (2005);
- GenElute Plant Genomic DNA Miniprep Kit (Sigma), Lucchetti et al. (2018).

In research conducted by Mafra et al. (2008) three different methods were tested for extraction of DNA from raw and processed soybean products: NucleoSpin Food (Macherey-Nagel), GeneSpin (GeneScan, now Eurofins) and Wizard (Promega). For the DNA extraction from soybean flour or products with high DNA content the most efficient were NucleoSpin Food and GeneSpin because both gave high purity and good DNA quantity, with the advantage of having the fastest and simplest protocols. For difficult samples and highly processed soybean foodstuffs (tofu, vegetarian foods, soybean desserts), the best results in terms of DNA quantity and quality were obtained with the Wizard protocols.

Comparing Nucleospin Plant (Macherey-Nagel), DNeasy Plant Mini (Qiagen), in-house CTAB-based and Wizard kits to extract DNA from honey, the best method in terms of DNA quality and amplification capacity turned out to be the Wizard method, combined with sample pre-treatment with a mechanical disruption step of pollen in order to improve the yield. Also, DNeasy and CTAB methods allowed amplification of the 18S rRNA and taxon-specific *adh1* genes, but extraction with these methods resulted in a lower DNA quantity (Soares et al., 2015).

The NucleoSpin Food Kit (Macherey-Nagel) gave the highest yield in almost all tomato-derived matrices, however, only the DNA extracted with the Wizard commercial kit (Promega) gave a PCR product of the expected size in all the analysed matrices, though the QIAamp DNA Stool Kit (Qiagen) provided the lowest degree of degradation (Turci et al., 2010). For rice-derived matrices, the Isoplant II Kit (Nippon gene) was compared to the Takara kit with magnetic trapping designed for uncooked dehulled and polished rice. The Isoplant II Kit (Nippon gene) is recommended for rice foods consigning of poor DNA quality due to processing and the Takara kit should be applied to rice foods rich in proteins (Sagi et al., 2009).

NucleoSpin Food Kit (Macherey-Nagel) used for DNA extraction from lamb and beef meats allowed the extraction of DNA with sufficient concentration, but samples contained PCR reaction inhibitors - Cq values for *tRNA-Leu* sequence were higher than plasmid Cq values (Sovova et al., 2017). In the same paper, a NucleoSpin Food Kit (Macherey-Nagel) and a DNeasy Plant Mini Kit (Qiagen) were tested for extraction of DNA from samples containing cranberries (jam and dried fruit). The amount of DNA extracted with both kits from both samples was sufficient, but only DNA extracted from jam with DNeasy Plant Mini Kit (Qiagen) was amplified in PCR (*tRNA-Leu* sequence). The dried cranberries contained various additives, such as citric acid or vegetable oil, which might have had an influence on the extraction process and caused the inhibiting substances to persist in the sample.

2.3.2.4 SDS-based methods

SDS-based DNA extraction methods are used by some EU labs for GMO testing. The principle of this method is to use a high concentration of SDS as detergent for cell lysis, followed by addition of chloroform/isoamyl alcohol (24:1) or phenol/chloroform/isoamyl alcohol (25:24:1) to remove non-DNA biomolecules such as proteins and lipids, and subsequent precipitation of DNA with isopropanol (Turci et al., 2010).

The advantages of the SDS-based method are that it improves cell lysis and ensures good removal of protein contamination from the matrix. It was considered the best conventional method for fresh tomato samples (Turci et al., 2010) and was validated for oilseed rape seeds (CRLVL07/04XP, 2017). A disadvantage is that SDS can be an inhibitor of subsequent PCR reactions. In fact, this method may require additional clean up (silica and 70% ethanol wash) as a possible action to eliminate the potential inhibitor (SDS) from the DNA sample (Terry et al., 2002). In the SDS protocol developed by Pirondini et al. (2010) DNA from milk and dairy products was purified by adding an extraction buffer containing 0.2 M Tris/HCl pH 7.5, 0.25 M NaCl, 25 mM EDTA, 0.5% SDS. The Cq values were lower for high lipid containing products (butter and Emmentaler cheese) compared to Cq values from the CTAB extraction method, which gave lower Cq values for all other matrices analysed (Pirondini et al., 2010).

Other protocol variations include decreasing the concentration of Tris/HCl pH 8 (0.01 M), NaCl (0.15 M) and EDTA (2 mM) and increasing the concentration of SDS to 1% (Turci et al., 2010) or combining this with freshly prepared PVP (6% of final volume) and one-half volume of 7.5 M ammonium acetate, added separately (Budelier & Schorr, 2001; Marmiroli et al., 2008).

There are not many variants of an SDS protocol adapted to kit format. The Nucleon Kit (Amersham), the WizardPlus and Wizard Magnetic from Promega are the few known examples.

2.3.2.5 Magnetic-beads separation methods

Magnetic beads-based methods are also known as solid phase extraction methods as the DNA binds to magnetic beads. These methods rely on the ability of DNA to bind and detach from a magnetic solid surface.

After DNA binding, beads are separated from other contaminating cellular components, washed and finally the purified DNA is eluted using ethanol extraction.

These methods are rapid and feasible for automation (e.g., with Maxwell instruments, Promega). However, they can be more costly than other methodologies (Jasbeer et al., 2009). An overview of the most well-known magnetic beads-based DNA extraction methods is shown in **Table 4** and described below.

Table 4. Basic commercial magnetic bead-based DNA extraction kits

Protocol	Matrices	Reference
Wizard Magnetic DNA Purification System for Food (Promega)	Dehydrated potato slices, flour, flakes, starch and flour. Processed food products; hazelnut; vegetables rich polysaccharides and polyphenolics. Highly processed soybean flour.	Smith et al. (2005); Coello et al. (2017); Lucchetti et al. (2018); Di Pinto et al. (2007); Corbisier et al. (2005)
KingFisher (Thermo Fisher) with MagneSilkF Genomic System (Promega)	Potato-based matrices	Smith et al. (2005)
MagNA Pure LC DNA Isolation Kit (Roche)	Feed highly processed (pelleted feed, expanded feed and coarse mixes)	Jasbeer et al. (2009)

The KingFisher instrument (Thermo Fisher) in combination with the MagneSilKF Genomic system (Promega) yielded the best DNA quality and was the preferable semi-automated procedure for potato-based matrices differentially processed and with different fat content. However, the Wizard Magnetic DNA Purification System for Food (Promega) was the best for dehydrated potato slices, flour, flakes, starch and flour with 20% fat (Smith et al., 2005). The Hi-Pure GMO Sample Preparation Kit (Roche) extracted much more DNA than Maxwell 16 tissue DNA Purification Kit (Promega) combined with the CelluACE XG system (Promega), but DNA was highly degraded. The Promega Kit on the Maxwell Instrument was considered as having a good performance (Sisea and Pamfil, 2007). Corbisier et al. (2005) describes the DNA isolation from highly processed flour containing GM soybean event GTS 40-3-2 using different extraction methods. They showed that DNA extraction from soybean flour subjected to excessive heat and vigorous mixing resulted in a statistically significant over-estimation of the GM-content when using a method based on magnetic beads coated with silica.

2.3.3 Most commonly used DNA purification methods

From the EU survey in 2017 (Annex III), approx. 61% of the participating labs indicated that they did not perform any additional clean-up after DNA extraction. However, for a lot of matrix types and depending on the downstream applications of the DNA extract, an extra DNA purification or "clean-up" step might be relevant. When additional clean-up is being conducted in the labs, the following protocols are applied most frequently: Qiagen QIAQuick (21%), Promega Wizard DNA clean-up resin (18%), additional ethanol precipitation (13%) and a lot of other, less frequently used methods (13%), e.g., Qiagen Genomic-Tip 20/G, Macherey-Nagel Genomic DNA Clean-up, Qiagen QIAEXII, alfa-amylase treatment (Annex III).

2.3.4 Limitations of DNA extraction methods

2.3.4.1 Impact of physical factors (industrial processing) on DNA degradation

Most food samples have undergone various levels of processing, including physical, chemical, and enzymatic treatments that can degrade and modify the DNA and protein within the sample. These may include prolonged heat treatment resulting in DNA degradation and protein denaturation, increased depurination and hydrolysis of DNA at low pH and enzymatic modification or degradation (e.g., nucleases and proteases) (Terry et al., 2002).

DNA extraction from charred samples (i.e., partially burned) is challenging due to inhibitors derived from the Maillard reaction. Silica binding columns are an effective method for extracting DNA from *Triticum aestivum* grains that were charred at 200 °C for 1 h. Pre-incubation of samples with N-Phenacyl thiazolium bromide (PTB) in a binding buffer prior to transfer to the silica binding column and prolonged washing of bound DNA with washing buffer, eliminated inhibitors in the DNA from charred samples (Giles & Brown, 2008; Sajali et al., 2018).

Extraction of DNA from processed canned tuna is challenging due to the filling media. In the study conducted by Chapela et al. (2007) and Sajali et al. (2018), four types of filling media were evaluated: oil, brine, tomato sauce, and vinegar. Among the filling media, tuna canned in oil produces a high concentration of DNA regardless of the DNA extraction method used. In addition, a high quality of DNA was obtained through the CTAB method.

Smoking, salt-curing, cooking, and other processing procedures for seafood could lead to DNA degradation. Tagliavia et al. (2016) reported rapid DNA extraction from various fresh, stored, and processed seafood products. The method replaced NaOH with KOH in the lysis buffer, eventually generating KCl following neutralization with Tris–HCl (Sajali et al., 2018).

2.3.4.2 Impact of chemical factors on DNA extractability

Di Pinto et al. (2007) compared two DNA extraction methods applied to a range of food products. The results highlighted a different efficiency in extraction and removal of the inhibitors interfering in the PCR test. The Wizard Magnetic DNA Purification System for Food (Promega) was reported as the most efficient approach for vegetable matrices rich in polysaccharides and polyphenolics. It proved to be simple and reliable for GMO detection; the use of a "mobile solid phase system", unlike a column-based system, provided the best PCR detection limit for vegetable matrices, probably due to their lower fat content and to higher efficiency in polysaccharides and polyphenolics removal. In contrast, the DNeasy Tissue Kit (Qiagen), modified concerning the lysis conditions depending on the size and type of the source material, demonstrated more efficiency with complex and processed matrices such as dumpling, chocolate snack, vegetable bouillon cube and cherry jam. The highly efficient DNA purification was probably due to the buffering conditions and silica-column-based system, which allow better DNA binding and removing of inhibitors. The sensitivity, the high specificity and the reproducibility of the DNeasy Tissue method suggest that it is feasible and ideal for routine analysis on dairy and meat products (Di Pinto et al., 2007).

2.4 Diversity of food and feed matrices

A food and feed matrix is described as a whole in which complex physical and chemical interactions of nutrients and non-nutrients and other compounds take place. It may be seen as a physical domain where the individual constituents provide new functionalities compared with their state when isolated or in a free state (Aguilera, 2019).

Processed food is justified by food industries to secure food (Thomas et al., 2018) but requires DNA technologies to authenticate the processed food (Campbell-Platt., 2017). Currently, the preferred methods for food authentication and also to detect GMOs, are DNA-based. However, even though DNA is the analyte of choice for all these methods, there is no single DNA extraction protocol useful for all the existing food matrices (Woolfe & Primrose, 2004; Piskata et al., 2019). Interestingly, Agbagwa et al. (2012) mention that "researchers continuously inundate the discipline with new DNA extraction procedures and modifications of existing protocols". Indeed, it seems that there is not one DNA extraction method that congregates all good features: fast, inexpensive, simple, not hazardous, producing good quantity of intact DNA.

The most reported difficulty in isolating DNA from certain food or feed is the weak recovery rate, mainly in complex matrices. Additionally, it is not always possible to reach the desired quality as stated by the CCMAS (2010). Due to the type and extent of industrial processing and the presence of components such as fat and sugar, currently, one of the greatest challenges imposed on laboratories is to recover DNA of enough quantity and with quality not inferior in terms of the size of DNA fragments to that present in the original matrix.

When the representativeness of the component or ingredient in the whole sample is very low (e.g., total pollen in honey varies from 0.003% to 0.104%, expressed in pollen mass relatively to the mass of honey, Piazza & Oddo, 2004; Kleinjans et al., 2012; Davison & Kershen, 2014), it will never be possible to recover high quantities of DNA from a certain material. According to the different types of matrices, DNA extracts are additionally affected by the presence of different residues and degradation forces which might interfere, to a different extent, with downstream applications. Among so many published protocols and results of DNA extraction experiments from different matrices, none provided information on the level of fragmentation during the technological processes used at the food industry. Moreover, all tested methods differed in their efficiencies in a non-reproducible manner. Therefore, there is no single advisable method per matrix (Piskata et al., 2019). In order to better decide upon the DNA extraction and purification methods to use and estimate the putative effects on the downstream applications, food/feed matrices may be grouped. According to Aguilera (2019) food matrices are classified as liquid, emulsion, gel, cellular, network exocellular, fibrous extracellular, viscoelastic, dense, porous materials and artificial. For our proposes, this classification is not appropriate as it

focuses on the physical properties of the matrices. For instance, plant materials are classified as "cellular matrices". We suggest a classification focused on the processing level and chemical composition with direct effects on DNA extraction, as follows:

2.4.1 Seeds and plant material without processing

Raw, fresh or dry, non-processed vegetable-derived food or feed refers to parts of plants, i.e., seeds, roots, tubers, leaves, fruits or other tissues gathered directly from nature. The following seed-based commodities, that may be relevant for GMO analysis, are typically distinguished as 'seeds from': grasses and legumes; crucifers and other oil or fibre species (such as soybean, oilseed rape and cotton); maize; sorghum; other cereals (such as wheat and rice); sugar and fodder beets (beetroot); subterranean clover and similar species; and vegetables, such as potato (OECD Seed Schemes, 2021).

Seeds are heterogeneous organs composed of different tissues. As they originate from a flower and result from the maturation of the fertilised ovule, their tissues are of different parental origin and may have different ploidy levels. DNA content, chemical composition (oils, polyphenolic compounds, polysaccharides, mucilage) and texture may vary considerably (Júnior et al., 2016) but, in most cases, this has no implication on DNA extraction. It is commonly reported that DNA extraction from seeds or from raw plant material is relatively easy. This was reinforced by the great number of simple and efficient protocols for DNA extraction from fresh and dry raw botanical extracts (Abdel-Latif & Osman, 2017; Salim Khan et al., 2007).

There are several exceptions, and it is not always possible to obtain DNA in the desired quantity and quality when taking into consideration the downstream application (Chabi Sika et al., 2015; Ragupathy et al., 2019). To overcome several issues, published literature cites hundreds of different protocols for DNA extraction from seeds and other plant material without processing (grains, tubers, various types of plant tissues and non-processed pure food or feed products). CTAB- and SDS-based protocols are usually the most applicable ones, although they incorporate many variations, often involving the use of several purification steps with hazardous reagents and special treatments for particular recalcitrant matrices. Most of the inhibitors can be removed from the DNA extracts with the addition to the extraction buffers of some reducing agents (β-mercaptoethanol) or PVP to remove polyphenols (Porebski et al., 1997), enzymes (e.g., α-amylase) or with a high salt concentration (Demeke & Jenkins, 2010).

While it may seem very simple to isolate nucleic acids from unprocessed food, there are a few issues that can be a problem:

(Hard) seeds or **rigid cell wall leaves** usually require disruption of the cell wall either with a dedicated mechanical process, for instance using a mortar and pestle, a grinding protocol, for instance using a ball or bead mill, or adding liquid nitrogen, pectinase, and/or cellulase (Manen et al., 2005) to the extraction buffer.

High oil (fat) content (e.g. soybean and canola seeds) interferes with the whole process as more purification steps with organic compounds (phenol:chloroform:isoamyl alcohol) are required. It may also be useful to treat samples with lipase or hexane (Demeke & Jenkins, 2010). All in all, this reduces DNA recovery and may result in co-precipitation with the DNA and inhibition of downstream PCR reactions.

High polysaccharides and **polyphenols** content (e.g., *Corymbia* and *Coffea,* cotton, sugar beet and fruits in general, potato tubers, rice, maize and wheat) may lead to difficulties in DNA extraction too. The interference during DNA extraction appears in different steps. For instance, mucilage interferes with the pipetting because it increases viscosity, therefore the volume of DNA extraction buffer has to be optimized and DNA quantity is usually strongly affected (Kundu et al., 2011); on the other hand, polysaccharides such as starch may co-precipitate with DNA inhibiting the enzymatic activity needed in the downstream applications; it may also alter the spectrophotometric measurements, thereby strongly affecting the estimation of the concentration and quality. Extracts of DNA contaminated with polysaccharides tend to degrade more easily (Healey et al., 2014).

High protein content: Several plant species (e.g., soybean**,** pea, clover and alfalfa) having protein-enriched seeds, such as those from beans (Fabaceae) are widely used in food matrices and are subject of routine GMO analysis in many countries. Additionally, for beans inclusive of soybean, there are several DNA extraction methods available (Aboul-Maaty et al., 2019; Agbagwa et al., 2012). Proteins may act as contaminants in DNA analysis however and should be removed during DNA extraction. CTAB works differently based on the ionic strength of the solution. At a low ionic strength, it precipitates nucleic acid and acidic polysaccharides, while proteins and neutral polysaccharides remain in solution. At high ionic concentration, CTAB denatures or inhibits the activity of proteins. NaCl helps to remove proteins that are bound to the DNA, and also helps to keep the proteins dissolved in the aqueous layer so they do not precipitate in the alcohol along with the DNA (Heikrujam

et al., 2020). Globular proteins get dissolved in water. To make them insoluble, their denaturation is one of the alternatives that can be done, e.g. by reducing intermolecular disulfide linkages with β-mercaptoethanol.

DNA content – Some cereal seeds like maize contain a triploid endosperm that has approximately as much extractable DNA as the embryo (Papazova et al., 2005). About half of the total DNA extracted from maize kernels originates from the endosperm and the other half from the embryo, although there is evidence that DNA quantity differs among cultivars. Besides varietal variation, differences in the homozygosity/heterozygosity/hemizygosity of these tissues will affect qPCR results since the zygosity level determines the number of target sequences.

2.4.2 Commercial processed plant products

DNA extraction methods used for processed samples may be different than those used for seeds and other non-processed plant material. During food processing, food components are released from their original matrices and converted into ingredients and combined into products (Aguilera, 2019). The aim is to increase their conservation, enable storage, increase the ability of being digested (cooking produces softer textures) and increase the consumer's satisfaction towards the consumption of e.g., "natural" and healthier food (Aguilera, 2019; Capuano et al., 2018). There are six food classification systems based on processing (Monteiro et al., 2019) among which, the NOVA system (Monteiro et al., 2010) is the most applied in scientific literature and adopted for this review (Monteiro et al., 2010). According to this NOVA classification we describe four groups:

Group 1: Minimally processed foods and feeds i.e., food altered by simple physical operations, e.g., washing, freezing/chilling, drying, milling/crushing/grinding, centrifugation, peeling and removal of inedible parts, roasting, boiling, pasteurization/vacuum packing, non-alcoholic fermentation. Examples are cleaned, frozen or vacuum-packed fruits and vegetables, dried grains or milled grains (flours, flakes, plain cornflakes), whole peeled tomatoes, non-refined oils, as olive oil, and honey.

Group 2: Processed ingredients result from foods from Group 1 transformed by processes such as pressing, refining, grinding, milling and drying. Examples are oils, butter, sugar and salt.

Group 3: Processed foods include canned vegetables, legumes, fruits (in syrup) and fish (in oil), freshly baked breads, simple cheeses. They have added salt, oil, sugar and other substances from Group 2. In this group of foods, the industrial processes are very simple and preserve the basic identity and most constituents of the original food.

Group 4: Ultra-processed foods are, for instance, snacks (sweet, fatty or salty), ready meals and products formulated from substances extracted from foods or derived from food constituents, carbonated drinks, cookies, pastries, cakes, margarine, breakfast cereals (sweetened), fruit yoghurt, pizza and pasta dishes, sausages, baby formulas, etc. Although these foods may result from the assembly of several food components (previously extracted from whole food), and differently processed foods and additives and ingredients of exclusive industrial use, it is the group most consumed. They result from the combination of intensive and diverse manufacturing processes involving different industries. Mechanical, thermal and chemical processes (enzymatic extraction processes, hydrolysis, hydrogenation, acidification, among others) are used to make products such as oils, starches, maize gluten, tofu, soy lecithin, wheat gluten and protein isolates, fried potatoes, tomato pulp, tomato puree. All of the mentioned treatments are expected to have an impact on the total DNA quantity and quality in the matrix. In this category (protein isolates: GROUP 4 Ultra-processed foods, https://www.fao.org/3/ca5644en/ca5644en.pdf), it is the expectation that only traces of DNA may be extracted, and these may be highly degraded. DNA extracted from highly processed food and feed samples is generally more sheared and of low molecular weight.

In some cases, no traces of the original unprocessed components are detectable. Some examples are food supplements e.g. probiotics, starter cultures, as well as some very particular food products like soy drinks, soy sauces, sausages, chocolate, pre-prepared food, sauces, etc.

To help laboratories in taking decisions concerning the most suitable DNA extraction method to apply to a certain food product, ultra-processed foods can also be grouped according to the most prevalent component: 1) fat-based products (cheese, butter/fats, oils and refined oils, margarines, nuts); 2) polysaccharides-enriched products (breads/pastries, pizza, biscuits/cakes, jams, sweet snacks, starches, sugar); 3) protein-enriched products (yoghurt, soya drink, soya derivatives, meat, fish).

Furthermore, animal feed from plant origin (examples are oilseed rape cake and soy meal) may be distinguished from animal feed from non-plant origin (meat or fish based). In addition, feed additives (e.g., choline chloride) are also considered as relevant (Annex III).

2.4.3 DNA extraction methods according to the matrix type

According to the International Life Sciences Institute report (ILSI, 1999), inhibition of the DNA polymerase during PCR reactions by the presence, in the DNA extract, of co-purified fats, among other molecules such as proteins, polysaccharides, polyphenolics, and other compounds present in processed and ultra-processed foods, is a major problem. For this reason, the ability to amplify the DNA obtained from food samples must be determined before conducting a GMO screening PCR assay or a GMO-specific identification or quantification.

Challenges for DNA extraction are highly heat-treated food products, hydrolysed plant proteins, purified lecithin, starch derivatives, and refined oils derived from GMOs (Pauli et al., 1998; Mafra et al., 2008).

2.4.3.1 Fat-based products

DNA extraction from food products being composed of high levels of fat or being involved/imbedded in fat (such as fish, seafood, fat-balls for bird feed and fish-based processed food products) may be more complicated due to the presence of fat. Fat removal is pertinent prior to DNA extraction. Oil and lipids may be removed from samples by soaking them in a mixture of chloroform/methanol/water (1:2:0.8) overnight (Piskata et al., 2017), pre-treating the sample with n-hexane (Žel et al., 2015) or extracting with phenol/chloroform/isoamyl alcohol (25:24:1) followed by precipitation with isopropanol at -20 °C and washing with 70% ethanol (Besbes et al., 2022).

2.4.3.2 Polysaccharide-rich products

The CTAB-based method is very versatile allowing for several modifications and it is by far the most used one for GMO analysis. For instance, an inexpensive CTAB-based protocol was tested on seeds and crops from 19 different species belonging to seven different plant orders (Aboul-Maaty et al., 2019). It uses a higher CTAB concentration and higher levels of β-mercaptoethanol, and higher concentrations of sodium chloride and potassium acetate were added simultaneously with absolute ice-cold isopropanol for the precipitation of DNA free from polysaccharides. These modifications consistently produced pure and high-quality DNA suitable for further molecular analysis (food safety, detection of GMOs, and biodiversity conservation). SDS-based protocols showed to be the more suitable ones for fresh tomatoes (Turci et al., 2010) while a CTAB-based protocol was the most efficient in extracting DNA from potato tubers (rich in starch) even if the quality of the extracted DNA was not high (Smith et al., 2005). Leaves of several plant species are composed of high levels of polysaccharides and polyphenolic molecules that co-precipitate with genomic DNA, giving to the DNA extracts a viscous, glue-like appearance. This consistency hampers a precise pipetting and can inhibit the downstream enzymatic reactions. Such polysaccharides (and also phenolic compounds) undergo rapid oxidation upon their release from leaf tissue and irreversibly bind to the phosphate backbone of DNA. The action of currently used DNA polymerases is strongly inhibited by these compounds and if the DNA is extracted, if without additional purification steps, it cannot be used in subsequent analysis (qPCR and high-throughput sequencing). The majority of DNA extraction methods from plant leaf tissue are derived from the original CTAB method (Doyle & Doyle, 1990). To overcome the problems associated with polysaccharides and phenolics, PVP, β-mercaptoethanol and high salt solutions can be incorporated in the protocol (Healey et al., 2014).

Starch, a complex carbohydrate, makes up nearly 80%–90% of the total weight of the rice grain. It is particularly difficult to get rid of the starch while extracting DNA from rice grains. This co-precipitated polysaccharide is known to inhibit DNA polymerase activity. Another problem with rice seeds are the husks (glume) of the grain. These are unextractable and when the standard CTAB protocol is applied the yield decreases dramatically if the sample intake is not increased. DNA extraction methods for rice often use seedlings or leaves rather than the grains and tend to be time-consuming, involve multiple steps, and use hazardous chemicals and expensive enzymes. Sajib et al. (2017) and Ashfaqur et al. (2017) describe a simple and affordable Chelex®-100 based DNA extraction method from rice grains. It does not require any hazardous chemicals or enzymes and extracts DNA with good purity indices (A_{260}:A_{230} and A_{260}:A_{280} values).

Although CTAB-based methods are very versatile, they have several potential drawbacks including higher risk of cross-contamination due to many pipetting steps, higher DNA degradation due to many centrifugation steps, and use of hazardous chemicals, e.g., phenol, chloroform, isoamyl alcohol, β-mercaptoethanol. Moreover, there is no possibility for automation, and the protocols are laborious and time consuming. Moreover, the presence of PCR inhibitors may not only originate from the matrix itself, but may also result from the reagents used for DNA isolation, e.g., CTAB at ≥ 0.005% (w/v), SDS at ≥ 0.005% (w/v), sodium acetate at ≥ 5 mM, sodium chloride at ≥ 25 mM (their concentration in the final extracts may be reduced by washing the ethanol precipitate with 70% ethanol). Alcohols, like ethanol ≥ 1% (v/v) or isopropanol ≥ 1% (v/v) may be removed by drying the pellet

and resuspending them in water or buffer. Another PCR inhibitor is phenol at > 0.2%, which can be removed by using PVP, PVP/ammonium acetate or addition of 1.2% citric acid to the DNA extraction buffer (Demeke & Jenkins, 2010).

Several commercial kits are available and were already tested to extract genomic DNA from plant tissues. They behave and affect the yield of amplifiable DNA differently for each of the matrices (Smith et al., 2005). Usually, the yield produced from commercial kits is low. This was already documented for bulked and single seed of cotton (Adnan et al., 2018). In an ILSI (1999) workshop report, an increased DNA quantity was obtained with a combination of the classical CTAB method and a subsequent DNA purification step.

Commercial kits can be applied to difficult-to-isolate plant species to supplement CTAB based extractions to generate genomic DNA of acceptable quality. Kit-based extraction methods operate efficiently for the removal of contaminants, but are often expensive, and the problem of losing DNA through subsequent column washes or precipitations can become important when only a small amount of matrix is available for a number of PCR analysis.

2.4.3.3 Protein-enriched products

In the paper by Stefanova et al. (2013) a modified CTAB method for DNA extraction from soybean and meat products was employed in comparative studies of DNA extraction methods for soybean detection in different meat products. The efficiency of the modified CTAB method was compared with three widely used commercial kits: GeneSpin (Eurofins); DNeasy Plant Mini (Qiagen), and Wizard Magnetic DNA Purification System for Food (Promega). DNA extraction from various processed (mechanical, thermal, chemical or enzymatic treatment) soybean and meat products was tested in order to find a reliable and efficient method for routine quality and authenticity control in the meat chain. The modified CTAB method produced the best results for DNA extraction from all tested products – soybean derivatives and meat products (Stefanova et al., 2013).

In a study by Piskata et al. (2019) the aim was to determine the degree to which DNA is influenced by the technological processes used in the meat food industry. Eight DNA extraction procedures were compared using self-prepared samples from both raw and heat-processed and/or mechanically treated muscles and different types of meat products and pet food (pork, beef, and chicken). It was found that the DNeasy Mericon Food Kit (Qiagen) was the optimal choice for the extraction of DNA from raw muscle, heat-treated muscle, and homemade meat products from multiple and single species. Technological processing significantly affected the yield and quality of isolated DNA molecules, as well as the ability of DNA to undergo PCR amplification. Regarding DNA quality evaluation, it was concluded that there was no relationship between concentration, A_{260}:A_{280} ratio, and the ability to undergo PCR amplification. Only PCR analysis can definitively establish the quality of the extracted DNA (Piskata et al., 2019).

Several techniques for extracting DNA from meat samples have been compared and reported by Yalçınkaya et al. (2017). Among the methods, the modified salt method from Cawthorn et al. (2011) was shown to be effective for extracting DNA from meat, producing intact DNA (Sajali et al., 2018).

Gelatin is predominantly composed of denatured collagen, with various amino acid compositions related to collagen. DNA extraction from gelatin has been achieved using nucleic acid binding column or standard ethanol precipitation methods. In addition, SureFood Prep Animal X (CONGEN Biotechnologie) had been reported to successfully extract DNA from processed gelatin food products. Shabani et al. (2015) reported a higher recovery of short DNA amplicons from gelatin-based products using DNeasy Mericon Food Kit (Qiagen).

Extraction of DNA from gelatin capsules was made possible by MasterPure DNA Purification Kit (Epicentre). Procedures were carried out according to the manufacturer's recommendations, except for some modifications such as addition of glycogen to assist DNA precipitation, addition of carrier tRNA to inhibit nonspecific absorbance of DNA and resuspension of DNA in nuclease-free water instead of a buffer supplied in the kit (Cai et al., 2012; Sajali et al., 2018).

2.5 Assessing the quantity and quality of DNA

Among the most frequently applied methods for DNA quantity controls after the extraction are spectrophotometric OD measurement (87%), qPCR, e.g., endogenous plant-target amplifiability controls (45%) and fluorometric OD measurements, e.g., with PicoGreen (13%). Horizontal agarose gel electrophoresis (AGE) and digital PCR (dPCR) are additionally mentioned (Annex III).

Accuracy and precision of DNA quantification by spectrophotometric OD ratio measurements can be influenced by (1) the size distribution of DNA in solution, (2) the ratio of ssDNA and dsDNA and (3) the presence of other

components that absorb at 260 nm, such as RNA and single nucleotides. For dsDNA, fluorometric OD measurements are more sensitive than spectrophotometry. Accuracy and precision of DNA quantification can be influenced by: (1) the size distribution of DNA in solution, (2) exclusivity of dyes (i.e., some dyes are more specific for dsDNA), (3) sensitivity of different dyes and their optimal working concentration ranges, (4) DNA integrity, (5) sensitivity of the assay to photobleaching and (6) DNA purity. Agarose gel electrophoresis enables semi-quantitative estimation of DNA concentrations by running serially diluted DNA of known concentration, and visually comparing the intensity of the fragment bands with the intensity of the bands of tested samples of unknown concentrations. This method also enables visualization, to some extent, of the degree of DNA degradation in the sample. See Chapter 3 for more details.

2.6 Challenges in DNA extraction for downstream GMO analysis

2.6.1 Impact of DNA extraction on analytically determined GMO content

It is known that DNA extractability depends on many factors such as type of matrix (degree of processing), particle size of the sample, homogenisation of the sample and DNA extraction protocol used. The case of food samples is typical: they are usually a complex mixture with several ingredients, sometimes treated with processes that degrade DNA. In this case the different treatments lead to a different extractability of the ingredients. For this reason, a classification of the food and feed samples based on the characteristics of the samples is important (see 2.5).

Particle size and homogenisation of the sample are crucial for obtaining a representative sample. The ENGL guidelines for sample preparation describe in detail the best practices to prepare samples from various matrices (Berben et al., 2014). The most efficient extractability is obtained from the finest particle sizes.

The selection of a DNA extraction method for a given matrix should not only be done considering yield and repeatability. In some cases, a different GM percentage may be obtained when the same sample is extracted with different extraction protocols. An example of such an experience from an ENGL lab in the past is the following. Two test items from a comparative trial composed of a compound matrix (soymilk powder) and a "raw" soybean flour respectively, were extracted with the preferred and validated method of the laboratory and with a silica column-based commercial kit. After fluorometric DNA concentration measurement and diluting the DNA extracts to the working concentrations, an inhibition test with a taxon-specific reference assay was conducted on the two extracts for each sample. Both "raw" and composite sample passed the inhibition tests. The GMO quantification runs showed a very good efficiency and R^2 parameters. The "raw" matrix showed no difference in the recovery when the two methods of extraction were used, while in the compound matrix a ten-times reduction in the recovery of the GM event was observed when applying the preferred validated method. As a result, the GM % was significantly different following the use of a different DNA extraction method.

This example shows that good amplification parameters and good inhibition tests do not always lead to a "true" value of the GM content in a sample. For this reason, considerable attention should be paid on this topic. The evaluation of the method of extraction should be done on various composite samples with different treatments before choosing a suitable method for a particular matrix. During this evaluation, different DNA extraction protocols should be applied on composite samples retained by the laboratory (e.g. proficiency test samples, samples labelled with a known concentration of analyte). Any deviation from the "true value" should be recorded and the method that gives the closest results should be chosen for the matrix investigated. A periodical investigation of the chosen extraction method on new samples is necessary in order to avoid errors in quantification of official samples.

2.6.2 Correlation between yield/amplifiability and matrix complexity

Amplification of DNA is more influenced by DNA quality and the overall structural integrity of the DNA compared to DNA concentration. Marmiroli et al. (2008) and Smith et al. (2005), observed differences in the amplification of different targets in the same DNA extract and this fact depended on the DNA extraction method used. The variation observed on DNA amplifiability of the reference endogenous genes and on the transgenic elements was not consistent.

This relation was also observed by Mafra et al. (2008) for soybean DNA extracted from different soybean derived food products. A lectin gene fragment in DNA extracted with the CTAB method was amplified in all samples. CTAB extraction as well as extraction with the kits NucleoSpin Food (Macherey-Nagel), GeneSpin (GeneScan), and Wizard (Promega) showed good PCR amplification for the soybean powder drink, but weak or non-detectable fragments in all the other drink samples. For the extraction of DNA from vegetarian foods only

CTAB and Wizard methods gave results. For the protein isolates and tofu, the range of detection limits was much higher than that for the soybean flours, where the lowest levels of detection were obtained with the CTAB followed by all of the kits.

Whereas the lecithin gene of soybean was always amplified (Marmiroli et al., 2008), variations were registered for the amplification of the *sus* gene, the endogenous reference assay for potato (Smith et al., 2005), as well as for the screening element CP4-epsps (Marmiroli et al., 2008). A similar observation was reported by Sagi et al. (2009) who concluded that DNA extracts may vary in composition as a result of the extraction method applied.

These observations are of very high importance as the consequences can be dramatic either on the screening for the presence of GMOs or on their quantification. Therefore, enforcement laboratories may have to implement additional DNA extraction methods to the methods that they currently apply to ensure that their results are reliable and comparable as described below.

2.6.3 Appropriate controls for DNA extraction

According to Table 1 in ISO 24276:2006, ISO 24276:2006/A1:2013, mandatory controls should be included in each step of a GMO analysis. As a minimum, a water extraction control (e.g., milli-MQ or equal, purified water instead of sample) as well as a positive extraction control should be included in each DNA extraction procedure in the lab. Appropriate reference materials should be used as positive control and/or calibrants in GMO quantification. Typically, a 1% (m/m) quality control material, or any other concentration in line with the expected GM content in the sample, is ideally extracted from a 1% (m/m) Certified Reference Material (CRM). For verifying if the DNA extraction procedure was carried out well, the negative and positive extraction controls are mandatory in one downstream qPCR, e.g., for qualitative detection of the species. The negative extraction control should give no amplification plot and no measurable Cq value in the species-specific PCR. The positive extraction control should result in a measurable signal (Cq) and normal amplification plot in qPCR. ISO 24276:2006, ISO 24276:2006/A1:2013 further depicts the expected outcome of the difference controls included during DNA extraction, as well as the solutions how to proceed in case of one or more of the controls are not as expected.

The CCMAS pays attention to the physical form of the CRMs, in particular the particle size that may affect the extraction efficiency of the target DNA, i.e., a smaller particle size increases the yield of DNA. The physical form of the CRM determines its suitability for use as positive control/calibration standards in qPCR analyses.

The CRMs and other control samples can be either DNA extracted from a matrix containing the analyte and certified for the level of presence, or plasmid DNA with the specific DNA target sequence. If none of these controls are available (e.g., in case of unauthorised GMOs), samples from proficiency testing schemes can be used, although there is no guarantee on the stability of the GMO content in such samples.

The reference materials and the controls should be treated in exactly the same way as the matrix to test, in terms of extraction procedure. This is because the extraction method may affect the recovery of the analyte. Records of the reference materials and other control samples should be retained. For more information on appropriate controls for DNA extraction, see also Chapters 3 and 4.

2.7 Conclusions and future challenges

The detection and quantification of an increasing number and diversity of authorised and unauthorised GMOs to be monitored is becoming more and more challenging.

DNA extraction is the first and crucial step in GMO identification and quantification, when using DNA-based analyses (e.g. qPCR, dPCR, NGS). DNA extraction methods, if possible, should be classified as suitable for a defined matrix. In this classification the results of ring tests and proficiency tests could be informative because they may be accompanied by a “true value” established and tested by the provider (if not determined by consensus between the participants).

The choice of the appropriate method for DNA extraction depends on the type of matrices and downstream application(s). Any method potentially used should provide a sufficient amount of DNA for amplification, of high quality and be free from impurities/contaminants that can inhibit the PCR reaction. The size of the extracted DNA (its structural integrity) should be larger than the amplicon produced during the PCR reaction. Different DNA extraction and purification methods have a significant influence on DNA quantity, quality, and estimation of the GMO content by qPCR. The different types of DNA extraction and purification methods used

may affect the results of the PCR amplification of specific GMO target sequences as well as endogenous, non-GMO target sequences.

The presence of GMOs is tested in raw plant materials as well as processed food and feed products. When choosing an appropriate method of DNA extraction, the characteristics of the matrix should be taken into account (e.g., the relative content of protein, fat, polysaccharide, or polyphenol), and there may be a need for optimizing the protocols. DNA extraction methods used for raw samples may not be suitable for processed samples or for different plant products such as seeds or flour. DNA extracted from processed food generally is more fragmented and has lower molecular weight than DNA from raw materials and pure ingredients. However, this may not necessarily affect the detection or quantification of GMOs. In principle, a DNA extraction method should ensure that the extracted DNA remains representative of the DNA composition in the (different ingredients of the) sample.

The CTAB-based method, with different additional steps and many variants (depending on the matrix), is the most widely used method after silica-column based protocols (most of these are commercialized and provided as a kit) and SDS methods.

The advantage of the CTAB and SDS methods over the ready-to-use DNA isolation kits is that they can be freely modified depending on the matrix and the type of impurities that need to be removed. A basic protocol can easily be modified by addition of enzymes such as proteinase K or other factors such as β-mercaptoethanol to the CTAB extraction buffer, or by changing the concentrations of the buffer components. All additional steps - washes and precipitation – generally decrease the overall yield of DNA but are useful for further purification of the DNA.

Currently, there is no universal method advocated for DNA extraction from all templates. The history of GMO analysis in many different labs in charge of official controls shows that **it is not possible to have one unique "all-purpose" DNA extraction method applicable to all food/feed/seed matrices**. It would be useful to develop an effective method for DNA isolation that automates the entire process, reduces costs and avoids contact with hazardous chemical agents while taking into account particular features of the matrix. Among the most challenging steps in routine GMO analysis is the assessment of the quality and quantity of the isolated DNA. To ensure successful downstream PCR amplification the purity of the extracted DNA is of a greater importance than the yield of DNA.

3 DNA quantity and quality check in routine analysis

3.1 Introduction: challenges related to DNA extraction

The DNA extraction is a critical step as it has to provide DNA with sufficient quantity and quality for subsequent qPCR analysis. ISO 21570:2005/A1:2013 requires that the test report has to indicate the DNA extraction method(s) used and also any deviations have to be mentioned. Analytical laboratories may encounter several types of challenges related to DNA extraction.

Challenges related to the matrices are e.g.:

- Diversity of the matrices: the laboratories performing GMO analysis need to analyse diverse types of samples, varying from simple seed, grain, flour or fresh fruit (e.g., papaya), tuber (e.g., potato), leaf (e.g., maize or petunia) to highly processed matrices.
- Complexity of the matrices: a sample can contain more than one plant species. Additionally, the ingredients of the sample can have been processed in a different manner and extent (e.g., soy meal, soy oil). Even within the same "matrix" the way of preparation might be different having consequences on the DNA extractability (e.g., different particle sizes due to use of different mills or freezing used for the leaves).
- Homogeneity of the matrices: The comminution of the particles for example increases the DNA quantity. A sufficiently small average particle size should lead to the uniform extraction of both GM and non-GM DNA from the various ingredients.

Low DNA quantity as well as poor DNA purity are big issues for an analytical laboratory.

DNA quantity: The extracted DNA has to be of a sufficient quantity for the downstream applications. The yield of the DNA extraction procedure for a given sample is usually known by the laboratory (except for the matrices analysed for the first time) and may differ between different methods. Sometimes, the DNA quantity is insufficient to perform all analyses. For instance, when GMOs are found present in the samples, further identification tests need to be performed, which may not be possible if the DNA quantity from one extraction is insufficient. The laboratory should have a procedure in place in case the amount of DNA is not sufficient for all the tests to be performed. A laboratory can have a documented procedure to double (or copy) the samples to be extracted for "low yield" samples, or in case of unknown matrices, to start from larger test portions. Or it could be necessary to repeat the same procedure either applying the protocol with small modifications or using a different method.

For some matrices it is impossible to obtain DNA with any extraction method. In such case, the outcome of the amplification of an endogenous target has to be declared as a negative result.

DNA purity: DNA extracted from samples with very low yield or from recalcitrant matrices (high oil or polyphenols or polysaccharides content) very often also have poor DNA quality, estimated in terms of optical density (OD). In this case, it is important to assess the quality for example with an inhibition test conducted on the extracted DNA. Such an inhibition test usually uses the amplification of an endogenous target gene on serial dilutions of the DNA extract, looking at the difference between the Cq values of 2 subsequent dilutions and evaluating if this difference is within the accepted limits. In most cases, the taxon-specific target methods for maize, soy, oilseed rape and rice are applied for most of the food and feed samples. However, event-specific assays can also be used.

DNA integrity: As PCR amplicons are relatively small, a certain degree of fragmentation of the extracted DNA is acceptable. It could be useful to estimate the DNA fragmentation as indicated in the MPR document (ENGL 2015). This is not a common practice during the routine analysis but may be part of the validation of a new DNA extraction protocol especially for recalcitrant matrices.

The routine analyses are performed for clients. A fast analysis service has to be guaranteed for the client of the laboratory and if a problem occurs during the analysis, this has to be resolved fast. Generally, if a failure is found during the routine analysis the extraction is repeated with the same or, if possible, with another method until DNA of a suitable quantity and quality is obtained. Each laboratory should have troubleshooting procedures in place how to deal with difficult and problematic samples (see also Chapter 6).

In this chapter we describe and give examples of the existing guidelines for quantity and quality control of the DNA, and their application in routine analysis. We provide technical information on the most widely used

methods to assess the quantity and quality of the DNA for further qPCR or droplet digital PCR (ddPCR) analysis. This information is useful for both experienced and non-experienced users.

3.2 Sample preparation

DNA extraction efficiency is unequivocally dependent on particle size. As the particle size in a sample decreases, the yield increases. When DNA quantity between maize samples of various particle sizes (i.e., flour, meal and grits) was compared, maize flour with the finest particle size provided the highest yield (Moreano et al., 2005). This observation can be easily explained by the fact that smaller particles offer a larger surface area of exposure to extraction reagents compared with larger particles.

It is important to obtain homogeneous samples prior to DNA extraction, therefore attention should be paid to this aspect. Grinding is not required in the case of a homogeneous GMO distribution throughout the sample and for liquid samples or those characterised by a small particle size. When the laboratory sample contains large lumps, a coarse grinding (or pre-grinding) is necessary as a first step. In a sample with a constitutionally heterogeneous particle size a comminution is required (chopping, crushing, cutting, blending, milling, pulverising). The following table lists food and feed matrices with homogeneous/non-homogeneous GMO distribution and requiring grinding and homogenisation (Italian National Control Plan for GM feed and Table 9 of "Guidelines for sample preparation procedures in GMO analysis" JRC 2014).

Table 5. Examples of food and feed matrices with homogeneous/non-homogeneous GMO distribution and requiring grinding and homogenisation

Major groups in food and feed			
Matrices	**GMO homogeneous distribution grinding not required**	**GMO homogeneous distribution grinding required**	**GMO non-homogeneous distribution grinding and homogenisation required**
Pasta, noodles	/	rice or maize pasta	/
Maize or rice grains, flours and creams	maize, rice flours and mixed flours, baby food, maize starch	/	maize popcorn, sweet maize, maize and rice grains
Bakery products, pastry products	/	biscuit, cracker, hardtack, cereal flakes, muesli, cereal ball	/
vegetables and derived	/	soy burger, tofu	mixed salads, cooked soy grains
dietary supplement	/	soy proteins, soy or maize bar	/
vegetable drinks and derived products	soy milk and drink, béchamel, soy yoghurt, soy fluid	soy cheese, tofu	/
roots, tubers	potato starch	/	potato
fruits	papaya juice	/	papaya
legumes, oleaginous seeds	all flours and oils	/	all seeds
simple feed	soy, maize, cotton, canola, sugar beet pulp	/	soy, maize, cotton grains or broken
complete feed, complementary feed	/	canola, sugar beet, potato, flax, rice	/
pelleted feed	/	variable mixed feed with soy, maize, cotton, canola, sugar beet, potato, flax, rice	
maize leaf	/	/	maize leaf (for a better grinding, freeze prior to grinding)

3.3 Pre-PCR control of the DNA quantity and quality

According to ISO 21571:2005 and ISO 21571:2005/A1:2013, quantity and quality checks of extracted DNA are performed by either physical (measure of absorbance at the specific wavelength), chemical-physical (use of intercalating or binding agents able to emit fluorescence), or enzymatic (bioluminescence detection) methods or by quantitative PCR. The latter method is especially suitable for composite matrices or for samples with low DNA content or whose DNA is degraded. It is for the user to choose the most appropriate method to be applied, depending on the amount and quality of DNA to be quantified and on the matrix from which the DNA has been extracted.

3.3.1 Parameters

DNA quantity/yield and quality evaluation generally comprises different parameters, which can be checked prior to PCR (pre-PCR), or by means of PCR. The following DNA quantity/quality parameters are described in the Method Performance Requirements document in agreement with ISO 21571:2005, ISO 21571:2005/A1:2013, ISO 24276:2006 and ISO 24276:2006/A1:2013.

DNA concentration: amount of DNA per volume unit of DNA solution. The DNA concentration should be appropriate for subsequent PCR analysis.

DNA quantity: total amount of DNA in the extract. The yield should at least be as much as is required for the subsequent PCR analysis. The DNA extraction method should provide similar yields for both GM and non-GM materials from the same matrix.

DNA Structural integrity: Breakage of genomic (high molecular weight) DNA into smaller DNA fragments. The minimum size of the majority of DNA fragments should be larger than the size of the amplicon produced by the PCR module used in subsequent analyses.

Purity: The absence of PCR inhibitors in a DNA sample.

The parameters and their acceptance criteria are explained in detail in the ENGL guidance on Method minimum performance requirements (ENGL, 2015).

3.3.2 Techniques

Accurate estimation of total amount of DNA is a critical component for many analytical processes involving nucleic acid, including various DNA manipulations and molecular analysis. Impurities in DNA can lead to inaccurate measurement of DNA concentration and could potentially inhibit downstream analysis. Moreover, several studies have highlighted the lack of standards which leads to difficulties in comparing results between different methods of quantification and from different laboratories (Bhat et al., 2010; Nielsen, 2008; Stevenson et al., 2005). The key challenges of DNA quantification include sensitivity (effectively measuring small nucleic acid amounts), accuracy (affected by purity, detection range) and specificity (dsDNA/ssDNA/RNA).

The techniques mostly used (see 2.5) are discussed in more detail in the following sections.

More information on the equipment that is frequently used for each of these methods is given in Annex IV.

3.3.2.1 Spectrophotometry

UV absorbance spectrophotometry remains one of the most common methods for the rapid quantification of DNA and RNA. The absorption properties of DNA can be used for detection, quantification and assessment of purity. For dsDNA that is typically intact, a solution with an A_{260} value that equals one optical density (OD) unit corresponds to a sample that contains 50 µg DNA/ml. For DNA that is largely degraded or consists of single-stranded DNA (ssDNA), an A_{260} value of 1 OD unit was found to correspond to 37 µg DNA/ml and 40 µg RNA/ml (ISO 21571:2005, ISO 21571:2005/A1:2013). For proteins in solution, an A_{280} value that equals 1 OD unit corresponds to a protein level of 1 µg/ml (Cavaluzzi et al., 2004; Stenesh et al., 1984).

The most common purity check for DNA and RNA is the A_{260}:A_{280} ratio. Pure preparations of DNA and RNA have A_{260}:A_{280} values of 1.8 to 2.0, respectively. If there is contamination with protein or phenol, this ratio will be significantly less than the values given above. If the ratio for DNA is higher than 1.8 this could suggest RNA contamination. In both cases an accurate quantification of the amount of nucleic acid will not be possible.

A number of substances absorb at 230 nm. This is the region of absorbance of peptide bonds and aromatic side chains. Therefore, an increase in absorbance at 230 nm can also indicate contamination, which may in

turn affect the 260 nm reading for DNA and RNA. Several buffer components (EDTA, TRIzol, HCl etc.) exhibit strong absorption at 230 nm and can alter the results of quantification. Low A_{260}:A_{230} values indicate contamination and may require re-purification of the DNA samples. For DNA an A_{260}:A_{230} ratio of 2 or above is indicative of a pure sample.

In general, the following OD ratios are considered as reference values for the evaluation of the DNA purity: $1.8 \leq A_{260}:A_{280} \leq 2.0$ and $A_{260}:A_{230} \geq 2.0$.

Although purity ratios are important indicators of sample quality, the best indicator of nucleic acid quality is functionality in the downstream application of interest (e.g., qPCR). For instance, DNA extracts with OD ratios indicating contamination with different substances does not necessarily lead to inhibition of the PCR and vice versa – inhibition can occur even in extracts with OD ratios within the acceptance limits.

Advantages and disadvantages

The main advantages of this method are the measures the absorbance of small volume samples, a wide detection range, quick and simple measurements and it does not require any other reagents or accessories. A disadvantage of this method is that single-stranded DNA (ssDNA) and RNA also absorb UV light at 260 nm and can therefore interfere with the results and cause overestimation of the double-stranded DNA (dsDNA) concentration. In addition, there is no information on integrity though it is known that nucleotides and small nucleic acids fragments still contribute to the 260 nm reading.

3.3.2.2 Fluorimetry

Fluorimetry is a method for quantifying dsDNA by using a fluorometer and a DNA-binding fluorescent dye. Fluorescence-based quantification is more sensitive than absorbance, particularly for low-concentration samples and the use of DNA-binding dyes allows more specific measurement of DNA than spectrophotometric methods. The use of fluorescence dyes to quantitate nucleic acid has become a common alternative to spectrophotometry (Singer et al., 1997; Jones et al., 1998). Hoechst bisbenzimidazole dyes, PicoGreen and QuantiFluor® dsDNA dyes selectively bind double-stranded DNA. The samples can be read in different supports such as tubes, cuvettes or microplates, depending on the quantity of the samples read in a quantification session. Fluorescence measurements are set at excitation and emission values that vary depending on the dye used. Most of the fluorometers require a standard curve for each quantification session. Preparations of serially diluted standards and test samples are incubated with a fluorescent dye such as Hoechst bisbenzimidazole or PicoGreen reagent. Fluorescence values of the standards are used to construct a linear regression against which the concentrations of the test samples are estimated by interpolation. In order to avoid certain dye degradation, the readings must be performed within a certain period. Some fluorometers will generate standard curves and calculate the concentration of samples, eliminating the need for manual calculations. As with absorbance methods, dilution factor must be taken into account when calculating DNA concentration from fluorescence values.

Advantages and disadvantages

Factors that mainly influence accuracy and precision of DNA quantification using fluorometry include: the size distribution of DNA in solution, exclusivity of dyes (i.e., some dyes are more specific for dsDNA), sensitivity of different dyes and their optimal working concentration ranges, DNA integrity, sensitivity of the assay to photobleaching and DNA purity (Singer et al., 1997).

PicoGreen® is a fluorescent nucleic acid stain that selectively binds to double-stranded DNA. However, PicoGreen® assay relies on external standards (e.g., Lambda DNA supplied by JRC Directorate F as ERM-AD442k) which has been quantified using A_{260} (Bhat et al., 2010; Stevenson et al., 2005). The fluorescent dye is added to the DNA samples, signals are read, and concentrations determined using a standard curve. It has an excitation maximum at 480 nm and emission of fluorescence can be read at 520 nm. Since PicoGreen® only binds to dsDNA, it has the advantage that the reported DNA concentration is an accurate estimation of the quantity of DNA that is present within in the sample and is not influenced by the presence of ssDNA or RNA. The assay is suitable for quantifying dsDNA prior to PCR and for quantifying post-PCR products but is not suitable for thermal cycling as when melting steps are included. PicoGreen® dye is sensitive to salts, especially divalent cations and ionic detergents. Some reports suggest there is as much as 94.6% decrease in signal intensity with PicoGreen® when genomic DNA contains 0.005% CTAB detergent (Holden et al., 2009).

Hoechst (bisbenzimide) dyes are sensitive stains and selective for dsDNA (under appropriate assay conditions), do not show significant fluorescence enhancement in the presence of moderate concentrations of proteins, and allow detection and quantification of DNA at concentrations as low as about 10 ng/ml. However, Hoechst 33258

requires high salt concentrations in the presence of RNA and low salt concentrations in the presence of ssDNA, suggesting that two different assay solutions are required to obtain selectivity when samples that contain both RNA and ssDNA are analysed (Labarca and Paigen, 1980; Singer et al., 1997).

3.3.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis is a routinely used method for separating proteins, DNA or RNA. Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The agarose concentrations usually used for quantification of genomic DNA are 0.8–1%. Any RNA, nucleotides and protein in the sample migrate at different rates compared to the DNA so the band(s) containing the DNA can be distinct. In order to visualize nucleic acid molecules in agarose gels, fluorescent dye (e.g., ethidium bromide or SYBR Green or new dye currently commercially available e.g., GelRed™ Nucleic Acid Gel Stain) are commonly used. Illumination of the agarose gels with 300-nm UV light is subsequently used for visualizing the stained nucleic acids. The presence of an intense, high molecular weight band in the gel indicates intact genomic DNA with minimal degradation. Degraded or sheared DNA and RNA contamination can be visualized as a smear towards the lower molecular weight portion of the gel (Shokere et al., 2009). DNA concentrations can be estimated semi-quantitatively by running serially diluted DNA, of known concentration, and visually comparing the intensity of the fragment bands with the intensity of the bands of test samples of unknown concentrations (Demeke and Jenkins, 2009) In addition to size separation, nucleic acid fractionation using agarose gel electrophoresis can be an initial step for further purification of a band of interest (Sharp et al., 1973).

Advantages and disadvantages

Gel electrophoresis is performed to determine the level of DNA fragmentation caused by the technological processes used in the food industry (mechanical, thermal, chemical, and enzymatic treatments). In addition, the chemical compounds present in food matrices (polysaccharides, proteins, collagen, polyphenols, fulvic acids, or lipids) may not be completely removed during the DNA extraction protocol and can affect the integrity of DNA (Piskata et al., 2017). Finally, some extraction methods lead to a higher fragmentation of DNA (Piskata et al., 2019).

The performance of gel electrophoresis is relatively time and labour consuming and requires presence of different types of equipment for running the gel and observation and recording of the results. In some cases, it might not be a part of the standard equipment of a GMO analysis laboratory.

DNA integrity is a parameter that needs to be considered especially when it concerns the validation of new DNA extraction method. The parameters and their acceptance criteria are explained in detail in Method Performance Requirement document (ENGL, 2015) and more details on its application is given in paragraph 4.2.3.

However, the integrity assessment is usually not a part of routine procedure. Assessment of DNA integrity is particularly important for processed or problematic samples. Both integrity and purity can be used to inform on the suitability of the extracted DNA for subsequent steps.

3.3.2.4 Analytical electrophoresis systems

Analytical electrophoresis systems can be applied as alternative of gel electrophoresis system. Hereunder several examples of techniques are given. It should be noted that this kind of equipment is not standard for laboratory performing GMO analyses.

3.3.2.4.1 Capillary electrophoresis system

Capillary electrophoresis performs high efficiency separation of large and small molecules including nucleic acids, through gel-filled capillaries with the use of high voltage. In contrast to conventional electrophoresis, capillary electrophoresis can provide high-resolution sample measurements and characterisation. Nucleic acids migrate through buffer solutions under the influence of electrical fields and separate based on ionic mobility (e.g., Agilent 7100 CE System, Promega Spectrum Compact CE System, Bio-Rad BioFocus 2000 and BioFocus 3000 system).

3.3.2.4.2 On-chip Microfluidic-based technology

On-chip microfluidic electrophoresis is based on the traditional gel electrophoresis principles transferred to a chip format. Microfluidic technology is used to pass 1 µl of sample through a micro-channel, which is filled with

a fluorescent dye and sieving polymer. When electrical voltage is applied, the sample moves through the channel which is imprinted onto the surface of the chip. Each micro fabricated chip contains separate wells (for samples, gel and the external standard – ladder) and an interconnected set of micro-channels used for separation of nucleic acid fragments based on their size as they are driven electrophoretically through it (Schipor et al., 2016).

3.4 PCR control of the DNA extracts

The isolation of the DNA may lead to the co-purification of substances that inhibit the PCR reaction. Inefficient PCR amplification may result from the use of an insufficient quantity of DNA due to the use of an inappropriate molecular weight or may be due to the presence of inhibitors in the DNA extract. Inhibitors in the DNA can reduce the efficiency and/or reproducibility of PCR and thus may contribute to inaccurate qPCR results (Demeke and Jenkins, 2009).

3.4.1 Testing for inhibition: guidelines

The application of PCR techniques on food, feed and highly processed matrices requires extraction and purification strategies designed to ensure an efficient recovery and the removal of the PCR inhibitors.

Guidelines for testing and evaluation of the absence of inhibitors are given in two documents: JRC technical report "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (ENGL, 2015) in the context of DNA method validation and in the JRC technical report "Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods" (ENGL, 2017) in the context of method verification. Application of ddPCR is less prone to inhibiting factors (Pecoraro et al., 2019).

Examples on the different approaches to perform inhibition test, based in this guide can be found in paragraph 4.2.4.

ISO 21571:2005 and ISO 21571:2005/A1:2013 also provides also some guidance for testing for inhibition. When establishing a new DNA extraction method, the presence of inhibitors in the extracted DNA may be estimated by using DNA spiked samples, i.e. adding a known internal control to the extracted DNA.

3.4.2 Application of the inhibition testing in routine analysis

3.4.2.1 Inhibition test by using serial dilutions

The inhibition control by using serial dilutions is the most frequently used method in the laboratories and it is applied for both qualitative and quantitative methods. The acceptance criterion is the difference (ΔCq) between the measured Cq value and the extrapolated Cq value of the undiluted sample used in the inhibition test.

Examples of the different approaches to perform inhibition test by serial dilutions can be found in paragraph 4.2.4.

3.4.2.2 Inhibition test by using spiking

Besides using serial dilutions, inhibition may also be assessed by spiking the PCR reaction with a known amount of an exogenous nucleic acid (e.g., a plasmid). The recovery of the exogenous nucleic acid is compared with the added amount. This procedure was proposed for the first time by Hoorfar et al. (2004) in a standard guideline for PCR testing of foodborne pathogens issued by CEN in collaboration with ISO.

The spiking assay can be designed in different ways. Hoorfar et al. (2004) reviewed two different internal amplification controls: competitive and non-competitive. A competitive internal amplification control is amplified with the same primer set (at least one primer) and requires the use of cloning techniques. With this strategy there is always some competition between the two targets and the amount of the internal amplification control measured may be affected by inhibition. This is influenced by the molar ratio, length of the products and secondary structures of both DNA fragments. Moreover, the rate of negative results in the target assays can be affected when a common primer is used for the amplification of the internal amplification control. Another drawback of the technique is a lower detection limit due to the competition between the assays.

When the target and the internal amplification control are amplified with a different set of primers the internal amplification control is not competitive. This type of assay requires a multiplex target PCR reaction. The inhibition target can be a sequence designed on a plasmid or another gene present in the tested DNA. The main drawback of this inhibition test design is that the target DNA needs to contain the inhibition target. Another

disadvantage is that the amplification of non-competitive sequences may not reflect the efficiency of amplification of the primary target.

3.4.2.3 Integration and consequences of inhibition testing in laboratory practice

These testing approaches can be integrated in different ways in the laboratory practice. Some laboratory experiences for the inhibition test on routine samples are summarised below.

The inhibition test is applied already at the screening analysis. In this case an endogenous reference sequence is targeted. Most of the laboratories use a dilution series including 2 to 4 points with 4 to 10-fold dilutions. Which reference sequence to target depends on the nature of the sample and the analytical workflow. For instance, all taxon-specific markers can be simultaneously tested in inhibition tests, or this could be done at the second step when the presence of a certain taxon is confirmed.

The target used for evaluation of the inhibition should be present in sufficient concentration. Deviation in the ΔCq could be due also to low target concentration.

When inhibition has been detected different measures can be taken before proceeding with further analysis, e.g., additional clean-up of the samples, repeating the DNA extraction with an alternative procedure, use of diluted samples and checking for inhibition for all screening markers. It should be noted that for the extraction of DNA from difficult samples most of the laboratories have previously tested and use an optimised procedure.

Finally, the observed inhibition might not have a strong effect on the qualitative analysis and can be neglected if it can be proven not to affect the qualitative result.

For GMO quantification, however, the inhibition can have a strong effect if it is observed for instance only for one of the targets. This can consequently have an effect on the calculated GM percentage. Similar to screening, the ΔCq method is usually used to evaluate the inhibition but applied on both event-specific and taxon-specific targets. Inhibition can be assessed for both the reference materials and the unknown samples. In case inhibition is observed, the following measures could be taken: to remove the points in the dilution series showing inhibition from the calculation of the final results, additional dilution of the samples, additional clean-up of the DNA or new DNA extraction with an alternative method.

4 Validation/verification

4.1 Introduction

Extracting DNA from homogenous and representative test samples is the first step in GMO analysis. DNA quality and quantity are critical factors that directly affect subsequent PCR analyses. To fulfil general requirements for the competence of testing, laboratories have to show that a method is fit for purpose by validation or verification (ISO 21571:2005, ISO 21571:2005/A1:2013, ISO/IEC 17025:2017). Thus, validation or verification is the prerequisite to use a method in routine operations.

4.2 Interpretation of criteria when validating a DNA extraction method

When implementing, validating or verifying a new DNA extraction method, a number of criteria that describe DNA quality and quantity and its suitability for downstream applications such as qPCR or dPCR have to be assessed. Generally, for qPCR and dPCR analysis the same DNA quality requirements should be applied (Pecoraro et al., 2019).

4.2.1 DNA concentration

DNA concentrations or amplifiable copy numbers of DNA extracts should be sufficient enough to perform PCR analyses that allow the analytical verification of requirements defined in Regulation (EC) No 1829/2003 and Regulation (EU) No 619/2011, for authorised GMOs and GMOs for which the authorisation is pending or has expired, respectively. For authorised GMOs, labelling is required at concentrations > 0.9% (m/m), while for unauthorised GMOs, there is a zero tolerance. For GMOs falling under Regulation (EU) No 619/2011, concentrations ≤0.1% (m/m) should reliably be detected. Thus, the practical LOD and LOQ for a GMO in a given DNA extract should be in compliance with these thresholds, of which 0.1% is the most stringent one.

Example: A PCR method has an absolute LOQ (LOQ_{abs}) of 40 target copies per PCR and an absolute LOD (LOD_{abs}) of 20 target copies per PCR. For analysing authorised GMOs at a practical LOQ (LOQ_{pract}) of 0.9 % (m/m), the concentration of DNA extracts should be high enough to measure at least 4,444 endogenous reference gene copies (containing ≅ 40 GM-target copies per PCR in an extract with 0.9 % (m/m) GMO). For GMOs with a pending authorisation under Regulation (EU) No 619/2011 the LOQ_{pract} should be 0.1 % (m/m), thus the DNA concentration of extracts should be high enough to measure at least 40,000 endogenous reference gene copies (containing ≅ 40 GM-target copies per PCR in an extract with 0.1 % (m/m) GMO). For unauthorised GMOs with a zero tolerance the practical LOD (LOD_{pract}) should be as low as possible. In this example, to measure around 20 target copies per PCR in 4,000 endogenous reference gene copies would mean to measure with a LOD_{pract} of 0.5 % (m/m); to measure around 20 target copies in 20,000 endogenous reference gene copies would mean to measure with a LOD_{pract} of 0.1 % (m/m). For most practical samples, especially when DNA was extracted from difficult or highly processed matrices or from complex samples consisting of several species, it is difficult to reach DNA quantities of 40,000 reference gene copies per reaction. In this case, GMO analytes with very low quantities should be at least detected qualitatively. If possible, the DNA extraction procedure should be optimized to yield a sufficiently high DNA concentration.

4.2.2 DNA quantity, extraction efficiency

DNA extraction modules should be fit for purpose and give yields that allow to perform a (limited) number of analyses. The yield should be sufficient to determine DNA quality and quantity as well as to perform qualitative or quantitative PCR analyses.

To keep a good balance between effort, time, cost and result, a laboratory should prefer DNA extraction methods with good extraction efficiencies. The extraction efficiency is calculated in microgram DNA per gram of the sample portion (Waiblinger and Grohmann, 2013). The extraction efficiency of a method could be checked, e.g., by spiking the sample with reference material. Assuming that the material of interest and the reference material behave similarly in terms of extraction efficiency, the analysis of the spiking materials (e.g., extraction efficiency checked with qPCR or dPCR) can provide information directly transferrable to the material of interest.

Example: A homogenised food sample is mixed with a known amount of (homogenised) reference material (e.g., CRM from JRC or AOCS or material with known composition of ingredients and % GMO from proficiency tests). DNA is extracted from this mixture and analysed by qPCR or dPCR. The extraction efficiency of the

material of interest can be expressed in relation to the (known) amount of reference material. Using this procedure, extraction efficiencies of different methods can be compared (Luber et al., 2014).

4.2.3 Structural integrity of DNA

DNA quality depends on the length, structural integrity and physical-chemical purity of the extracted DNA. It can be checked by gel electrophoresis on a conventional agarose gel or by microcapillary electrophoresis (see also 3.3.2.3-3.3.2.4). In most qPCR or dPCR methods DNA-targets of <200 bp are amplified. Therefore, a DNA extraction method should yield DNA-fragments that are larger than 200 bp or at least the average targeted amplicon-size, assuming that this is present in the matrix.

Structural integrity of DNA can also indirectly be analysed by qPCR or dPCR. If a qPCR or dPCR results in positive amplification for a distinct target gene, then the DNA extract contains DNA of the respective target-size or larger. However, it cannot be excluded that the presence of relevant amounts of smaller DNA fragments affects amplification of different PCR targets. If a relevant amount of DNA fragments containing the GM target is smaller than the PCR amplicon size, it would not be detected, and GM quantification would result in underestimation of GM content.

Example: The event-specific method for quantification of GTS 40-3-2 soybean targets a reference gene with an amplicon size of 74 bp and an event-specific sequence with an amplicon size of 84 bp. For reliable PCR analysis of GTS 40-3-2, a DNA extract should consist of fragments larger than 100 bp.

4.2.4 DNA purity

The first information on how pure a DNA extract is, can be determined by spectrophotometry (see also 3.3.2.1). Moreover, DNA extracts should show no inhibition at relevant concentration levels. The choice of the target assay to test inhibition may affect the outcome. In some cases, a GM target may be more sensitive to inhibition than the taxon-specific gene of the event-specific detection method. For critical samples, it may therefore be worth testing both the GM and reference targets for absence of inhibition. This may, however, be difficult if the GM target is present in a low copy number resulting in unreliable Cq values in the dilutions measured. For dPCR applications, inhibition is generally reduced to a minimum due to the dilution of the sample.

Example: DNA is extracted with the same method from multiple test items of a processed food matrix. Concentrations of DNA extracted are determined by spectrophotometry. Although DNA yield is sufficient for all extracts, some extracts show ratios A_{260}:A_{280} < 1.8 indicating impurity. However, inhibition tests show that slopes of the inhibition curves lie between -3.6 and -3.1 (80 % - 110 %), R^2 are >0.98 and the difference between the theoretical and the measured Cq-value for the highest concentration level are <0.5. Together, all extracts are fit for purpose.

4.3 Approaches for method validation

Method validation should give objective evidence that a newly developed DNA extraction method, purchased kit or literature method provides DNA suitable for GMO analysis. In their daily routine, control laboratories usually face different situations that have to be addressed experimentally.

4.3.1 A method is used for the first time

If a laboratory wants to use a DNA extraction method for the first time, it has to show that the method is fit for purpose. However, depending on previous data available, the scientist should decide if an **in-house validation** or **verification** is necessary.

In-house validation is recommended if there is no previous data available for the DNA extraction method of choice which will be applied for the matrix of relevance or a similar one. This can especially be the case, when a DNA extraction method should be used for very specific or difficult matrices like soybean lecithin (Waiblinger et al., 2007), maize starch (Waiblinger et al., 2012a) or pollen isolated from honey (Waiblinger et al., 2012b).

As part of the performance assessment of a DNA extraction module, it is recommended to process the given DNA extraction protocol on different days (e.g., 3 days) with an adequate number of test portions (e.g., 6 per day), to obtain suitable data for the evaluation of the module. For in-house validation it is recommended to use a factorial design, thereby testing different conditions (e.g., increase/decrease the input per test portion, vary buffer volumes, include/exclude enzymatic treatments, etc.). After checking DNA quantity and quality,

different parameters like repeatability, reproducibility and robustness of the DNA extraction module can be determined. Further details can be found in the Example in Annex I.

Method verification is recommended for DNA extraction methods that have previously been validated on the same or a similar matrix or product (by in-house or ring-trial validation or during kit development of a manufacturer). This is the case when using an extraction method already published (publishing in known journals usually require a comprehensive validation) or introducing a standard method. An extraction method, that has successfully extracted DNA with sufficient quantity and quality from maize kernels could for example be used to extract DNA from rice or wheat kernels. For respective DNA extraction methods, DNA is extracted two (better three) times, each time on two test portions, if possible, on different days and by different operators (see Verification of analytical methods for GMO testing when implementing interlaboratory validated methods). Thereby the laboratory has to determine whether the method delivers DNA of suitable quality and quantity for subsequent analyses.

Table 6. Comparison of sample numbers and experimental repetitions needed for in-house validation or verification

No. of test-items	Type of validation	No. of independent test portions per sample	No. of independent extractions
12	In-house Validation	6	3
4 – 6	Verification	2	2 – 3

4.3.2 A new commercial kit is used for a distinct set of matrices

There are numerous manufacturers that offer commercial kits for DNA extraction from different types of matrices. Usually, the extraction methods have been validated on a distinct set of matrices by the manufacturer (e.g., DNA extraction from food including chocolate products, breakfast cereals, muesli, cookies, cakes, etc.). Although the manufacturer's procedure of method validation is usually unknown, commercial kit methods can be treated like in-house validated methods. Thus, it is recommended that laboratories that want to use a commercial DNA extraction kit perform a **method verification** for the designated matrices.

If a commercial kit should be used beyond the scope described (e.g., use DNA extraction kit tested on environment and stool samples for food products), an **in-house validation** should be performed. Alternatively, a **method verification** including additional positive control samples that have previously shown to be compatible with the chosen extraction method have to be included.

4.3.3 Minor modifications are introduced to an existing extraction method

If a laboratory wants to introduce minor modification, that have not been tested during in-house validation, or wants to expand the scope of an existing extraction method, it is recommended to perform a **method verification**. Minor changes could include:

- modification of buffer volumes (e.g., for absorbing or welling matrices)
- modification of test portion
- addition of solvent extraction-step (e.g., Chloroform, n-Hexane)
- addition of enzymatic treatments (e.g., RNase, α-Amylase, Cellulase)
- extension of time for enzymatic treatments

By adequately verifying the modified method, the laboratory has to show that the performance is as good as the original method.

Another modification could be the expansion of the scope by introducing a new matrix. Also here, a verification, showing that sufficient DNA quantity and quality can be achieved, is recommended.

4.4 Proposing a method as national or international standard method

In specific cases a laboratory or a group of laboratories might agree that a distinct extraction method is of major importance for overall routine analyses. As a consequence, the extraction method might be proposed to become a national or international standard method. For DNA extraction methods that are intended to become

standard methods an **inter-laboratory validation study** has to be performed. Inter-laboratory validation studies provide valuable information about the general applicability of methods and are important steps towards method harmonization. However, this report emphasises that such standard methods will never be applicable to all types of samples.

Although inter-laboratory validation studies give a valuable set of information on method performance, it is not necessary for every single method. Since they are very time- and cost-consuming, a careful consideration should be given prior to performing an inter-laboratory validation study.

An example of a detailed procedure that includes planning and implementation of an inter-laboratory ring-trial is described in Annex II.

5 Decision support systems

5.1 Rationale for establishing decision support systems for DNA extractions

GMOs are tested in various sample types – food, feed, seeds and plants from the field. Each sample may contain only one ingredient, e.g., maize seeds, or a number of ingredients, such as feed composed of a variety of grains, e.g., maize, rice and soybean (compound feed). Additionally, particular processed samples, such as cornflakes, can differ depending on the manufacturing procedure resulting in DNA being present in low amounts and partially degraded. Therefore, different DNA extraction methods are used depending on the different sample types.

The selection of methods for DNA extraction and purification is mostly done on experience-based choice of the user, as noted in ISO 21571:2005 and ISO 21571:2005/A1:2013 that deals with nucleic acid extraction for detecting GMOs. Several extraction methods have been proposed in this standard, together with their scope and examples of samples for each method. Others were developed or modified for specific matrices.

Different bioinformatics decision support system tools are commonly used for screening for GMOs in the EU using PCR-based approaches (Wilkes et al., 2017).

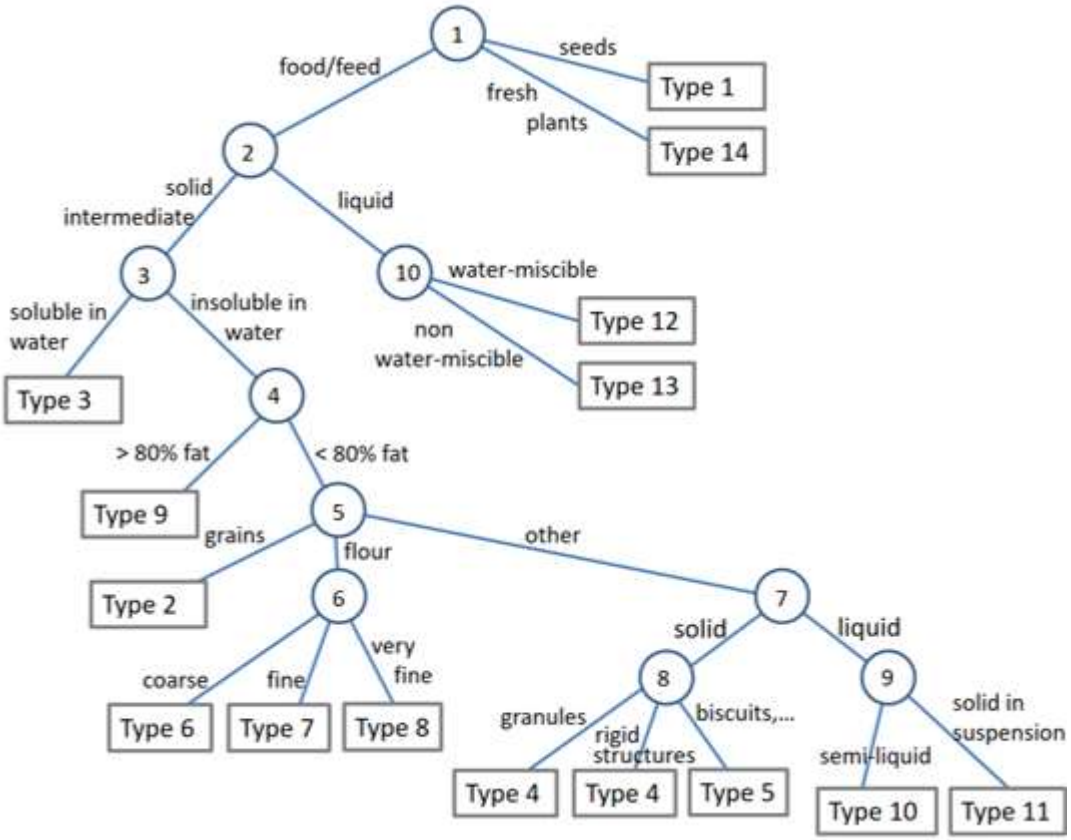
Using different extraction methods for different samples may not be cost effective and can be time-consuming for laboratories testing many samples daily. It is therefore essential to establish, in the laboratory, a manageable decision support system (DSS) that can support selection of the extraction method appropriate for an individual sample. The advantage of such a system is that different samples coming to the laboratory can be processed in parallel using the same method, thus resulting in rationalisation of the workflow, labour and associated costs. Consequently, fewer methods need to be verified or validated and the personnel training is more rational. Nevertheless, laboratories usually develop a system, where for most samples one or two different extraction methods are used.

Some laboratories are already using a DSS. Existing DSSs are based on different approaches of extraction method selection. The preferred choice for applying a specific DNA extraction method for a specific matrix can already be formalised in an SOP or is 'common knowledge' in a laboratory. Nevertheless, it is advisable to formalise these choices in a DSS in order to clarify and harmonise the rules of engagement within the laboratory. A DSS is not static; whenever a new matrix is to be analysed, the DSS can be extended. Most approaches are based on the selection of the extraction method depending on the matrix and/or downstream molecular biology applications. A DSS can also include determination of DNA quality and quantity. All different approaches are presented in the next section of this chapter.

5.2 Some decision support systems currently in use

At the Walloon Agricultural Research Center (CRA-W, Belgium), an identification key was developed to allow the type of matrix to be defined (Debode et al., 2021). Methods of sample preparation and DNA extraction are then associated to each type of matrix. The identification key works with a fixed sequence of identification steps, each with multiple alternatives, to make the choice for the next step. Going through the different steps, a decision tree is built up and results in different possible categories of matrices. The full architecture of the identification key proposed is presented in the Figure 1. Each question leads to a type of matrix or to a subsequent question that will permit 14 matrix types to be defined. Each matrix type is associated with a sample preparation, one or several DNA extraction methods and a recommended test portion. The identification key is useful at two levels. Firstly, technical staff in charge of the analysis can easily sort out what methods to associate with the sample for its preparation and DNA extraction. Secondly, to prove to the auditors of accreditation bodies that the different steps of the analysis whatever the sample are well controlled and that each type of matrix can be characterised.

Figure 1. Architecture of the identification key to decide the matrix type at CRA-W, Gembloux, Belgium



At the Institute for Agricultural, Fisheries and Food Research (ILVO, Belgium), in the routine laboratory for GMO analysis, three different protocols for DNA extraction are in the scope of accreditation (see Figure 2). The three protocols have been validated on several types of matrix/sample and the lab has many years of experience in applying the three protocols for different types of products. Based mainly on this experience, a DSS for DNA extraction has been set up, outlining the basic principles that will influence the choice between the three extraction protocols.

The most important criterion is the composition of the sample. For pure and single-species plant samples, such as e.g., seeds and pure plant materials, as well as minimally or non-processed food/feed samples with clear plant traces, DNeasy Plant Mini Kit (Qiagen) is applied. Typically also, this protocol is applied for dry matrices.

In case if a food/feed sample has undergone moderate to extensive processing steps AND/OR contains many different species as ingredients (thus mixed or composed samples) AND/OR is not dry but liquid/high-viscous, the Nucleospin Food Kit (Machery-Nagel) is the preferred kit for DNA extraction. Examples are e.g., drinks, sauces, prepared food meals, complex composed feed samples. Also, typically "difficult" matrices like e.g., test items in proficiency tests for GMO analyses, matrices potentially containing GMMs, etc. are subject to the Nucleospin Food Kit (Macherey-Nagel).

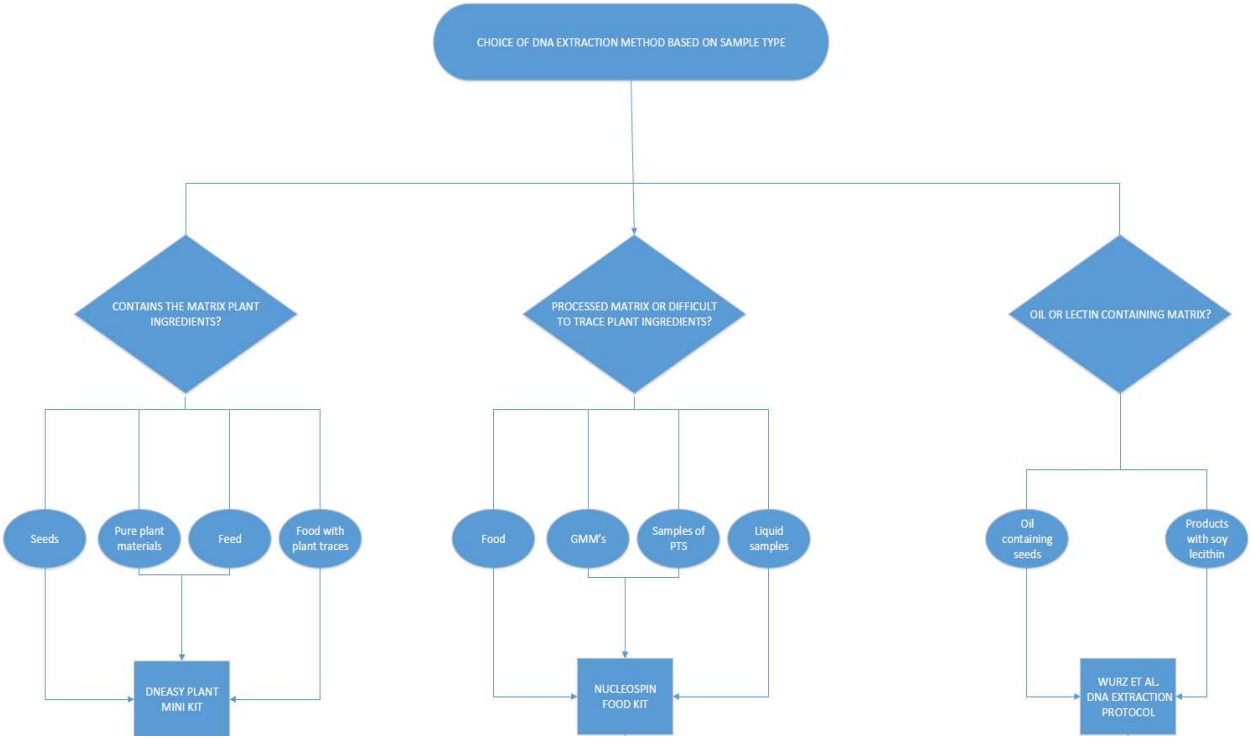
When selecting an applicable DNA extraction protocol in routine GMO analysis, the main issues will be whether the sample has been processed or not on the one hand, and whether it is single species/pure or not on the other hand. Based on these two questions, the DNeasy Plant Mini Kit (Qiagen) or Nucleospin Food Kit (Macherey-Nagel) is chosen.

In addition, there are a few particular matrices (exceptions to the matrices for which DNeasy Plant Mini or Nucleospin Food Kit can be chosen) containing oil or soy lecithin, where the DNA extraction protocol described in Würz et al. (1998) is applied. Examples here are canola or linseed (sowing) seeds containing high levels of oil, and chocolate or other products containing soy lecithin. Again, here, extensive practical experience in the lab plays an important role.

Each DNA extraction protocol has its own amount of starting material: 100 mg for DNeasy Plant Mini (Qiagen), 200 mg for Nucleospin Food (Macherey-Nagel), 2 g or ml for the protocol described by Würz et al. (1998).

Modifications to the DNA extraction protocols are possible, based on knowledge on particularities regarding the composition or nature of certain samples. Examples here are highly recalcitrant samples like e.g., canola cake or linseed cake from the feed industry, but also canola certified reference material powders where e.g., 100 mg instead of 200 mg starting material is weighed before the DNA extraction. A lower amount of starting material has proven to be an efficient adaptation to the Nucleospin Food DNA extraction protocol for these samples.

Figure 2. DSS for selection of DNA extraction protocol primarily based on sample composition at ILVO, Belgium



At Wageningen Food Safety Research (WFSR, Netherlands), first a short DSS (see Figure 3) and then, if necessary, an extended DSS (see Figure 4) is applied to monitor animal feed (food, (compound) feed, ingredients) for the presence of GMOs. On a case-by-case scenario modifications like hexane treatment, heating, washing steps etc. are also required. Other matrices like *Chinese rice*, vitamin-B2 require alternative approaches.

Figure 3. Short DSS applied to monitor animal feed for GMOs at WFSR, Netherlands

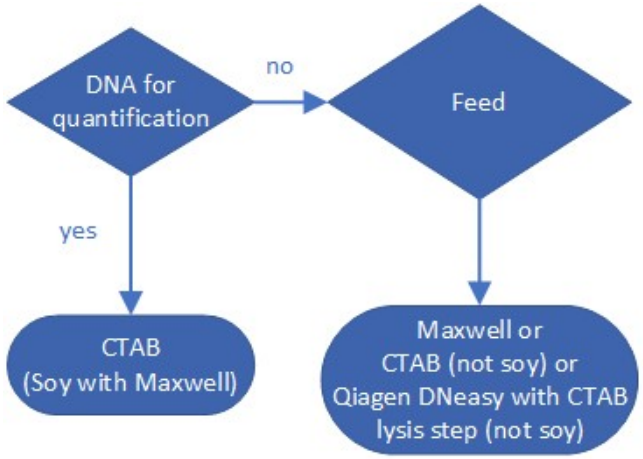
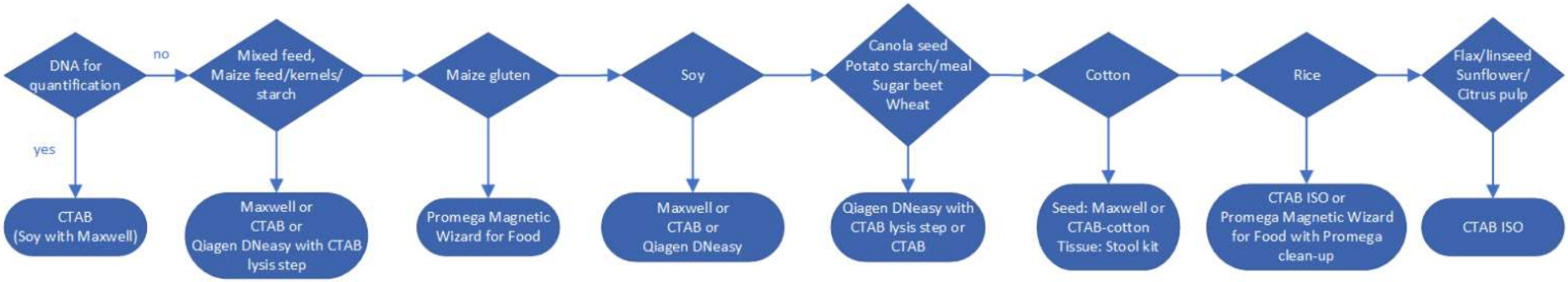
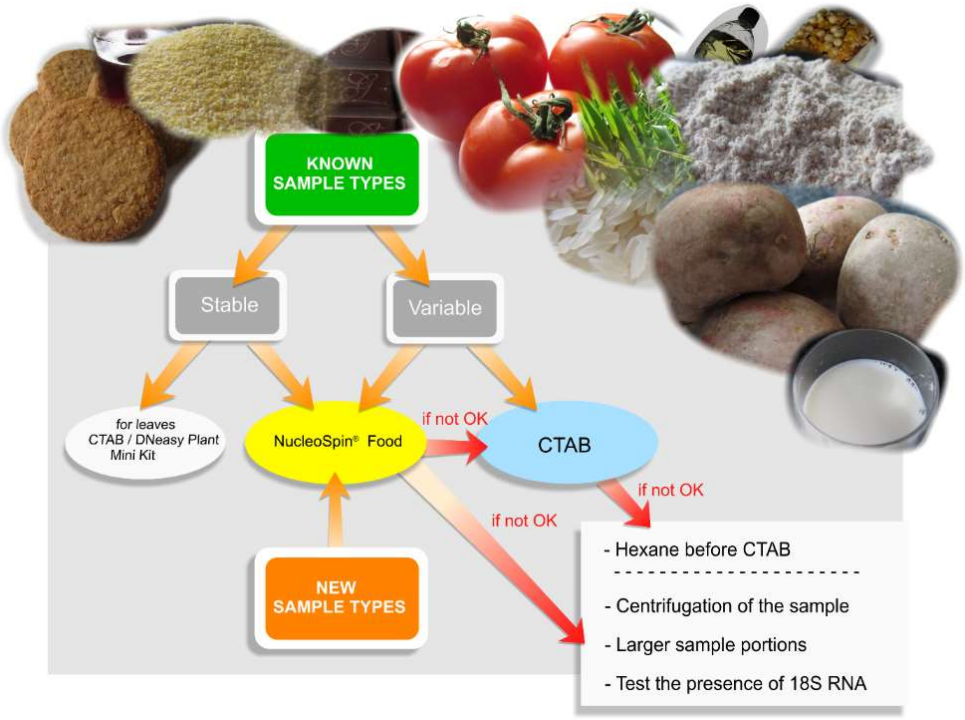


Figure 4. Extended DSS for different food and feed matrices at WFSR, Netherlands



At the National Institute of Biology (NIB, Slovenia), a DSS (see Figure 5) for selecting extraction methods for GMO testing on different samples - food, feed, seeds and whole plants was established based on many years of GMO testing experience, where various extraction methods have been tested for individual samples. The previous knowledge of the sample type was taken into account. Samples, tested many times previously with knowledge regarding DNA extraction regarding efficiency, were termed **Known** sample types. They were divided in two groups, one where repeatable results of DNA extraction can be expected, regardless of the producer of the sample, named **Stable** sample types, and **Variable** ones, which gave unexpected results of DNA extraction. Samples in which there is no experience with DNA extraction were named **New** sample types. NucleoSpin Food Kit (Macherey-Nagel) and the CTAB method (Annex A.3, ISO 21571:2005) were used for extracting DNA from most samples. Additionally, the DNeasy Plant Mini Kit (Qiagen) was used for extracting DNA from leaves. For more challenging samples, some additional modifications of the extraction procedure were introduced based on practical experience and are presented in Žel et al. (2015).

Figure 5. DSS for selection of DNA extraction methods at NIB, Slovenia



At LGC (UK), DSS's are in place for DNA extraction, which are based on determination of DNA quality and quantity (Figure 6). A full detailed explanation can be found at Science Search (defra.gov.uk), https://www.youtube.com/watch?v=2Ad5d5lm9FM&feature=youtu.be and in Wilkes (2019).

Following DNA extraction from a sample, the absorbance at 260 nm is evaluated. For absorbance values <0.1, low recovery of DNA is inferred, and the sample can either be re-extracted or concentrated (for example, using ethanol precipitation and resuspended in less volume, or using a microcentrifugation column).

If an absorbance value at 260 nm of 0.1 or above is obtained, then the next step is to evaluate the A_{260}:A_{280} absorbance ratio, an optimal value often being in excess of 1.8. Values <1.8 can be indicative of protein and/or phenol carryover. Corrective actions that can be implemented include re-purification of the sample or re-extraction with the inclusion of a proteinase K step in the case of suspected protein contamination.

Provided the criterion for the A_{260}:A_{280} ratio is satisfied, an aliquot of DNA can be subjected to electrophoretic evaluation. The lack of a smear and the presence of a bright high molecular weight band below the loading well are indicative of a primarily intact DNA sample. If the sample is marked by the presence of a characteristic smear ("comet-tail"), then this infers that the isolated DNA is fragmented and degraded, and re-extraction can be considered, particularly so should the DNA be associated with unprocessed food samples.

The next step involves evaluation of the absorbance ratio at the 260 nm and 230 nm wavelengths. Ideally, values for this should be in excess of 1.5. Readings below this value are indicative of the presence of phenolate ions, salts, and polysaccharides, which may inhibit any downstream PCR reaction.

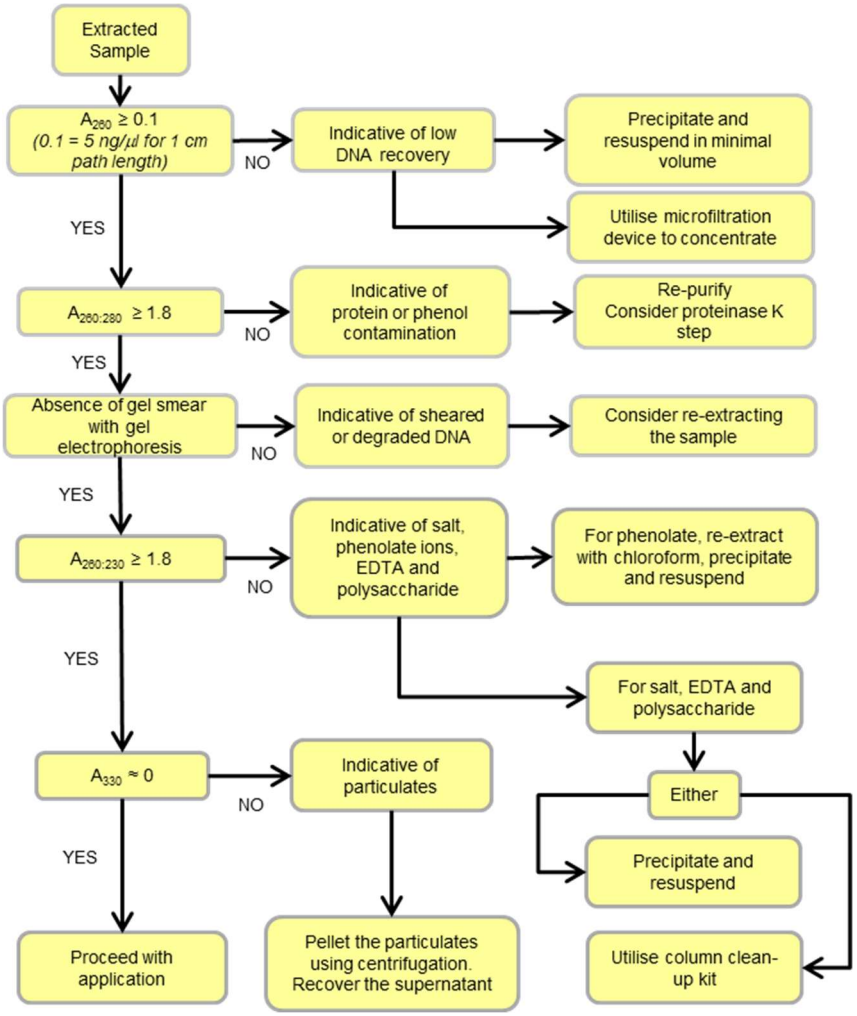
Corrective actions that can be undertaken in the case of suspected phenolate ion contamination include either re-extraction with a new sample or chloroform extraction of the original DNA solution. The latter is then followed by ethanol precipitation of the aqueous phase to recover the purified DNA and resuspension of the resulting DNA pellet in an appropriate buffer.

Should contamination with salts be suspected, the DNA can either be purified by ethanol precipitation followed by resuspension, or alternatively, the salts can be selectively removed using a commercial clean-up column.

Once the A_{260}:A_{230} ratio criteria has been met, the absorbance at 330 nm can be measured. Values greater than zero are indicative of particulate contamination and can originate from a number of sources. However, this is usually rectified with use of a short but high *g* centrifugation step to pellet the particles and transfer of the DNA solution to a new tube.

Should the absorbance value at 300 nm be approximately zero, along with the above qualifying criteria being passed, this generally indicates that the DNA extraction has been successful, and the sample can usually proceed for further downstream analysis using the appropriate molecular biology method.

Figure 6. Example spectrophotometric and gel based decision tree used at LGC, UK (EVID4 Evidence Project Final Report (Rev. 06/11) published by DEFRA (Science Search (defra.gov.uk))



5.3 Selection approach for challenging samples

Some samples can be very challenging to extract DNA from since they are highly processed and/or DNA is present at very low amounts or is degraded. Additionally, in some samples, contaminants and inhibitors may be present which can inhibit the PCR reaction. For such difficult samples, it is ideal to apply modifications or additional steps to pre-existing methods already included in the DSS, instead of developing additional methods, in order that the routine working process is not interrupted or challenged further. Some suggestions for these modifications are listed below.

When testing of the final product is proving difficult, it is advisable to ask for examples of the raw material if available, because the extraction of DNA from the raw material is usually more successful.

When testing the effectiveness of DNA extraction by a method targeting the reference gene, the possibility exists that the extraction from a sample with several ingredients was effective, but that insufficient reference gene was present. Since preferred reference genes are usually chosen based on their being only one copy present in the genome, alternative targets characteristic for all eukaryotic cells that can also be present as multiple copies (e.g., the 18S rRNA gene), can be additionally used to test the success of DNA extraction.

Certain additional steps can be used to minimize inhibition, such as the addition of proteinase K to degrade proteins or of *n*-hexane to remove lipid components (Gryson, 2010; Solfizzo et al., 1998; Terry et al., 2002).

Extraction of DNA from oil samples is also difficult, because the DNA remains only in the leftovers of plant material present in the oil. Therefore, prior centrifugation of the sample, using the sediment for further extraction can be helpful (Costa et al., 2010).

Some examples of DNA extractions from challenging samples are in Annex V.

6 DNA extraction trend analysis – collective experiences of ENGL members

6.1 Introduction

In June 2017, the EURL GMFF, with the assistance of LGC (UK), organised a three-day workshop on DNA extraction, held at the JRC (Italy). The workshop was attended by over 30 experts representing 19 EU member states as well as Mexico, Ecuador and Brazil.

The workshop consisted of a number of seminars and interactive discussion sessions on different approaches and quality control criteria for DNA extracted from food and feed samples. The workshop was designed to capitalise upon the shared knowledge and collective expertise and experiences of the ENGL and other scientists working in the topical area of extracting DNA from challenging matrices.

Based on the output of the EURL GMFF training workshop, a table of ENGL member experiences on DNA extraction from GMOs was collated. The resulting "DNA Solutions Table", listing issues and solutions commonly encountered when extracting DNA from different matrices, was made available to ENGL members through the ENGLnet (under Forum on DNA extraction, only accessible by ENGL members).

The aim of the DNA Solutions Table was to provide readily accessible information regarding experiences of DNA extraction from food and feed samples, to be used as a reference for ENGL members. Nonetheless, it was recognised that the sharing of these experiences on extracting DNA from challenging matrices would benefit the wider analytical community. Therefore, this current chapter was written in order to identify some of the major trends associated with those experiences and provide general recommendations on DNA extraction for GMO analysis.

6.2 Application of the trend analysis

This trend analysis was conducted on the current DNA Solutions Table (available to ENGL members), with the aim of identifying common DNA extraction issues and their associated solutions. Example common trends were identified using various filters associated with column descriptors (column headings) labelled as "Issue" and "Specific techniques", as applied to the DNA Solutions Table correct as of 28/10/2020. These trends were by no means-exhaustive, but the results provide information on the most common issues identified with DNA extraction approaches, as well as associated ways to resolve these issues, and therefore provide valuable insight and guidance in the area.

Information in the DNA Solutions Table was categorised and sorted according to the following two column descriptors:

- "**Issues**" - this descriptor referred to specific problems associated with DNA extraction identified by a laboratory, grouped according to eight general headings (Low yield of DNA extracts; Methods and protocols; Known problematic matrices; Poor purity of DNA extracts; Requirement of additional clean up; Observable PCR inhibition; Lack of observable PCR amplification; Loss or lack of DNA).
- "**Specific techniques**" - this descriptor referred to the specific technique linked to the problem which can occur during DNA extraction. This was usually characterised based on the method or the commercial kit used for the extraction.

Results of these searches and the filters are shown in Tables 7 and 8.

6.3 Results and discussion

The information housed within the DNA Solutions Table provided identifiable trends based on recorded "Issues": these related to specific problems associated with DNA extraction identified by a laboratory, which tended to group into several distinct headings (referred to above and also in Table 7). Grouping the table entries according to this descriptor resulted in the following identifiable trends:

The largest proportion of issues submitted on the DNA Solutions Table concerned **low yield** of DNA extracts, accounting for 29% of the total entries. This appeared to be mostly associated with either kit specific issues or general DNA extraction principles. Solutions to the issue of low yield were varied but predominantly included altering the sample size or choosing an alternative extraction kit.

Samples with **known problematic matrices** (e.g., those with high fat content) also presented a recurring issue, accounting for 17% of total entries in the DNA Solutions Table. Most of these were associated with the application of in-house CTAB DNA extraction approaches. There were no universal solutions for this, the tailored solution being dependent upon the test sample matrix content (e.g., addition of Proteinase K for samples with high protein content).

Equally well, issues associated with **methods and protocols** were a common occurrence, also accounting for 17% of total entries in the DNA Solutions Table. Once more, the majority of the entries related to application of in-house CTAB DNA extraction approaches. Solutions to help improve the in-house CTAB DNA extraction approach included additional stages in the extraction process, use of additional reagents and increasing the lysis buffer volume.

Poor purity (e.g., as observed by sub-optimal absorbance values) and **observable PCR inhibition** associated with DNA extracts were other common issues. Poor purity of DNA extracts was often corrected for by using additional clean-up stages (e.g., chloroform washes, ethanol precipitation, etc.) or by using a reduced elution volume. PCR inhibition was also commonly treated through using additional clean-up stages as described above, but also through diluting the DNA template.

Lack of observable PCR amplification as an issue was often successfully treated by increasing the sample input amount prior to extraction or by diluting the extracted DNA.

Loss or lack of DNA following extraction appeared to represent only a minor concern as a reported issue. Successful solutions to this issue included consistency of orientation of the sample tubes in the centrifuge or centrifuging at 4 ˚C for a prolonged period of time.

Repeating the DNA extraction with an alternative method (in-house CTAB with minor modifications depending on the matrix or using a different kit) was the most common solution for different issues e.g., problematic matrices, low yield of DNA extracts or observable PCR inhibition.

The DNA Solutions Table also provided identifiable trends based on recorded "Specific techniques": these usually related to the use of a specific method or commercial kit used for DNA extraction. Grouping the table entries according to this descriptor resulted in the following identifiable trends:

The use of **in-house CTAB DNA extraction approaches** represented the highest proportion of entries associated with the descriptor of "Specific techniques", accounting for 40% of cases. Solutions to help improve the results from in-house CTAB approaches included additional stages in the extraction process, use of additional reagents and increasing the lysis buffer volume.

Generic principles represented the next most common issue, representing 26% of total entries. These were further subdivided into the following groups:

- **Low yield** had a number of proposed solutions associated with it, common ones including increasing the sample size or using an alternative extraction kit;
- **Problematic matrices** were resolved using specific treatments e.g. addition of Proteinase K to treat samples with high protein content;
- **Lack of PCR amplification** appeared to be commonly resolved by increasing the amount of sample intake, or by further diluting the extracted DNA;
- **PCR inhibition** appeared to be mainly resolved through diluting the original DNA stock solution, or by the application of additional clean-up stages following the DNA extraction.

Absorbance values associated with extracted DNA outside the optimal range accounted for 10% of total entries. Solutions successfully applied to resolve the issue included repeating the wash stages, additional purification, and addition of clean-up stages.

The **NucleoSpin**[M] **Food Kit** (Macherey-Nagel) and related DNA extraction kits (https://www.mn-net.com/nucleospin-food-mini-kit-for-dna-from-food-740945.50 (cited 24/08/2022)), which was one of the most popular DNA extraction kits, representing the last identifiable trend, accounting for around 9% of total entries. Entries included some reported instances connected with low yield, inhibition or low supernatant volume after lysis. These were often solved by an additional CTAB stage prior to using the kit protocol, use of an amylase stage prior to cell lysis, application of a chloroform wash following lysis or by increasing the lysis buffer volume.

Overall, a number of issues were identified, many of these focussing on the use of the in-house CTAB DNA extraction approach, specific kits and protocols, samples with known problematic matrices, low yield and poor purity of DNA extracts, and presence of observable PCR inhibition. Whilst solutions to these were often bespoken and tailored to the actual issue, many of the reported issues appeared to be successfully resolved through combinations of the following common solutions: increasing the sample size; choosing an alternative extraction kit; application of an additional CTAB stage prior to using the kit protocol; addition of Proteinase K for samples with high protein content; use of additional reagents; use of an amylase stage prior to lysis (for matrices containing high starch levels); application of a chloroform wash after lysis; increasing the lysis buffer volume; additional stages in the extraction process (e.g. additional ethanol precipitation stage); and/or diluting the DNA template.

All of these potential solutions are in line with best measurement practice in the field of DNA extraction and purification principles (as described in Chapter 3 "Validation/verification" and Chapter 4 "DNA quantity and quality check in routine analysis").

The majority of trends and issues identifiable from analysis of the DNA Solutions Table were mainly associated with entries based on the application of in-house CTAB DNA extraction approaches. It is the Working Group's view that this demonstrates the popularity of using in-house CTAB DNA extraction approaches for routine DNA extraction compared to other methods, as well as how well the approach has been characterised and is understood, as supported by the number of proposed solutions. Easily identifiable solutions associated with some of the issues commonly associated with the in-house CTAB approach included using additional stages in the extraction process, use of additional reagents and increasing the lysis buffer volume.

A similar trend was observed with the NucleoSpin Food Kit (Macherey-Nagel), where the number of issues/solutions reported provide evidence supporting the routine use of this commercially available kit. Reported issues included low yield, and inhibition or low supernatant volume after lysis. Solutions offered included application of an additional CTAB stage prior to using the kit protocol, use of an amylase stage before lysis, application of a chloroform wash after lysis or by increasing the lysis buffer volume.

Table 7. Summary of DNA Solution Table entries sorted according to the "Issue" descriptor

DNA Solution Table entries sorted according to issue					
No. of entries	**Issue**	**Sample type**	**General Approach**	**Specific technique**	**Solution**
17	Low yield of DNA extracts	A range of different sample types	All associated with the extraction method	Either kit-specific or related to general DNA extraction principles	Solutions were varied but included altering the sample size or choosing an alternative extraction kit
10	Methods and protocols	A range of different sample types	Predominantly extraction methods and a single CRM reference	Associated with a range of extraction approaches, predominantly manual CTAB	Solutions included additional stages in the extraction process, use of additional reagents and increasing the lysis buffer volume
10	Known problematic matrices	A range of different sample types	All associated with the extraction method	Most entries related to observations using the manual CTAB method	Bespoke solutions for each issue e.g., addition of another reagent in the extraction
7	Poor purity of DNA extracts	No predominant sample type	Predominantly associated with DNA quantification approaches	Observations mostly associated with absorbance values	Solutions included repeated chloroform washes or clean-up stages, ethanol precipitation and reduction in elution volume
5	Requirement of additional clean up	A range of different sample types	All associated with the extraction method	Mostly associated with general DNA extraction principles	Solutions include repeating the washing step or performing an additional clean-up stage (e.g., with a kit or a further phenol/chloroform wash)
5	Observable PCR inhibition	A range of different sample types	Mostly associated with DNA extraction method	No predominant extraction approach	Solutions included diluting the DNA and using additional clean-up stages
2	Lack of observable PCR amplification	Soya milk and maize snack	Both associated with the extraction method	Both associated with general DNA extraction principles	Increase the sample input or dilute the extracted DNA
2	Loss or lack of DNA	Not associated with any sample type	Both associated with the extraction method	Both associated with manual CTAB only	Both solutions associated with adaptation of the centrifugation step

Notes:
The issue "Requirement of additional clean up" showed similar entries to the "Poor purity of DNA extracts" entries.
The issue "Lack of observable PCR amplification" showed similar entries to the "Observable PCR inhibition" entries.

Table 8. Summary of DNA Solution Table entries sorted according to the "Specific Technique" descriptor

DNA Solution Table entries sorted according to specific technique					
No. of entries	**Specific technique**	**Sample type**	**General Approach**	**Issue**	**Solution**
23	CTAB	A range of different sample types	Almost all associated with the extraction method, but just one with a CRM reference	Whole range of issues related to DNA extraction by CTAB protocol	Solutions were very varied with no identifiable trends
15	Generic principles	A range of different sample types	All associated with the extraction method	Associated with low yield, problematic matrices, inhibition or lack of PCR amplification	Solutions included increasing the sample amount, additional purification, diluting the DNA template, repeated washes or clean-up stages
6	Absorbance values	A range of different sample types	DNA Quantification	A_{260}:A_{280}/$_{230}$ are outside the optimal range	Repeat clean-up, washes, additional purification, choose DNA dilution with lack of inhibition to PCR
5	Nucleospin	No predominant sample type	Extraction method	Low yield, inhibition or low supernatant volume after lysis	Solutions included increasing incubation time, additional CTAB stage before using kit protocol, amylase stage before lysis, chloroform wash after lysis, increase lysis buffer volume

7 Conclusions

DNA is the preferred target analyte for GMO detection as it is stipulated that GMO control is to be performed with DNA-based methods, such as (q)PCR. Therefore, the quantity and quality of the extracted DNA is pivotal for optimal downstream analyses. In order to provide guidance for the National Reference Laboratories (NRLs) and Official Control Laboratories (OCLs), the ENGL mandated the Working Group (WG) on DNA extraction.

One of the aims of this technical document was to collect and describe the different DNA extraction methods, as well as provide guidance on several common issues and how these methods can be optimally applied. In this study, the different options for DNA extraction were sourced from literature and from methods that are currently being applied in ENGL laboratories.

Since official control laboratories have to show that a chosen method of modification is fit for purpose, validation or verification is a prerequisite for use of the method in routine analysis. This document gives guidance on choice and number of test samples for validation or verification of an extraction method in the context of different situations. Besides implementing a method in the laboratory, introduction of technical modifications is often necessary.

Overall, it can be concluded that there are many variations on the theme of DNA extraction, and there is not one protocol that is the preferred choice for a laboratory. Additionally, there is also no single DNA extraction protocol that works effectively across all food and feed matrices. The optimal method of choice needs to be determined on a case-by-case basis. Acknowledging the diversity of food and feed sample matrices as well as the plethora of DNA extraction approaches, this guidance document draws upon expertise from analytical laboratories to provide example practical solutions to DNA extraction issues which are commonly encountered. Implementing a decision support system (DSS) may help in rationalizing the workflow, labour and costs in the laboratory.

This guidance document provides the basis for a more informed choice on suitable DNA extraction methods, providing a valuable resource for all analytical laboratories to benefit from.

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Annexes

Annex I. Example workflow for in-house validation of a honeybee product-specific extraction method

1. Selection of samples

For method validation, different honeybee products were selected: Propolis, Royal gelly, Beebread, Pollen from black locust, lime, rapeseed, sweet chestnut, 13 unifloral honeys from Austria:

#	Honey sample
N1	Honey with pollen from wildflowers (polyfloral)
N2	Honeydew honey with pollen from conifers
N3	Honey with pollen from sweet chestnut
N4	Honey with pollen from sweet chestnut
N5	Honey with pollen from black locust (*Robinia pseudoacacia*)
N6	Honey with pollen from black locust (*Robinia pseudoacacia*)
N7	Honey with pollen from rapeseed (*Brassica napus*)
N8	Honey with pollen from sunflower (*Helianthus annuus*)
N9	Honey with pollen from lime (*Tilia*)
N10	Honey with pollen from lime (*Tilia*)
N11	Honey with pollen from rhododendron (*Rhododendron* spp.)
N12	Honey with pollen from wildflowers
N13	Honey with pollen from dandelion

2. Determination of DNA-concentration and -yield

DNA-concentration and -yield measurements were solely done with pure pollen samples: sunflower pollen, dandelion pollen and buckwheat pollen.

Extraction of sunflower- and dandelion pollen was performed in 6 replicates, on 2 different days respectively. Buckwheat pollen samples were extracted in 3 replicates on 2 different days, due to a limited amount of sample.

All extractions were performed under repeatability conditions. DNA concentration of extracts was determined by fluorometric measurement. An excerpt of results is shown in .

Figure A1.1.

Figure A1.1. Excerpt of results generated by fluorometrical measurement of DNA concentrations.

	sunflower		sample intake
1.	0,984	µg/ml	0,04g
2.	0,86	µg/ml	0,04g
3.	1,03	µg/ml	0,04g
4.	0,924	µg/ml	0,04g
5.	0,644	µg/ml	0,04g
6.	1,38	µg/ml	0,04g
	0,97		**mean value**
	0,24		**standard deviation**

	dandelion		sample intake
1.	< 0,010	µg/ml	0,003g
2.	0,696	µg/ml	0,003g
3.	1,42	µg/ml	0,003g
4.	0,572	µg/ml	0,017g
5.	0,6	µg/ml	0,007g
6.	0,756	µg/ml	0,008g
	0,81		**mean value**
	0,35		**standard deviation**

	buckwheat		sample intake
1.	2,56	µg/ml	0,03g
2.	1,75	µg/ml	0,025g
3.	1,08	µg/ml	0,009g
	1,80		**mean value**
	0,74		**standard deviation**

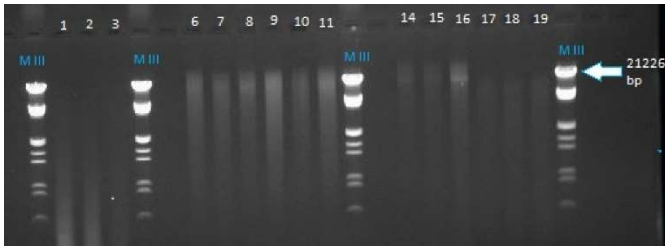
Measurement by flourometer
yields are low but DNA is amplifiable in PCR

For all samples, DNA-yields are very low. The DNA amount, however, is sufficient for PCR analysis (PCR amplification successful). Due to very low sample amounts the calculation of the mean extraction efficiency (µg DNA per gram sample) was not feasible.

3. Determination of structural integrity

Structural integrity of extracts was assessed by agarose gel-electrophoresis directly after DNA extraction (Figure A1.2).

Figure A1.2. Picture of agarose gel. MIII, DNA ladder with upper band (~21,226 bp) indicative of high molecular weight DNA. Sample 1-3: buckwheat pollen, Sample 6-11: sunflower pollen, Sample 14-19: dandelion pollen



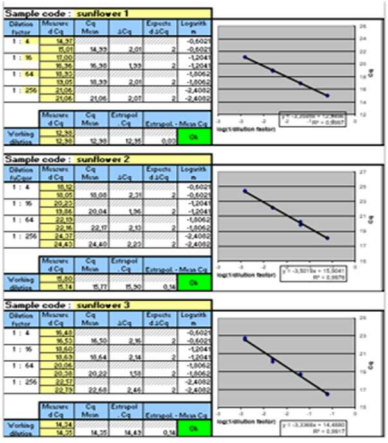
For all pollen extracts, DNA of high molecular weight (Bands >21,226 bp) can be detected. Extracts from sunflower and dandelion pollen contain a higher amount of high molecular DNA fragments than extracts from buckwheat pollen.

4. Determination of purity by PCR-inhibition testing

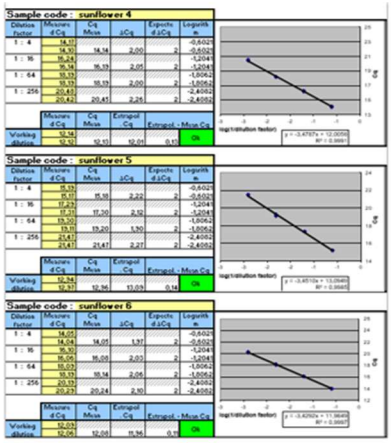
Inhibition testing was done with 1:4 diluted DNA-extracts according to ENGL criteria defined elsewhere (see ENGL Guidance documents for method verification or Minimal Performance requirements). Inhibition was tested for all pure, non-processed materials (pollen).

Figure A1.3. Excerpt of inhibition tests performed for extracts from sunflower and buckwheat pollen. Results for inhibition tests on DNA extracts from sunflower pollen (A, B), and buckwheat pollen (C). Summary of the results (D) .

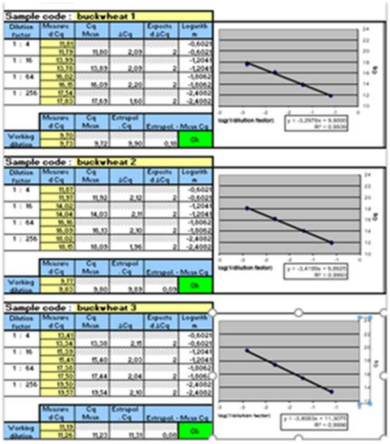
(A)



(B)



(C)



(D)

Results summary

Comparison of extrapolated Cq values versus mean of measured Cq values

Sample code	R^2	Slope	Cq extrap	mean Cq measured	ΔCq	
sunflower 1	0,9997	-3,358	12,95	12,98	0,03	Ok
sunflower 2	0,9976	-3,502	15,90	15,77	0,14	Ok
sunflower 3	0,9917	-3,337	14,49	14,35	0,14	Ok
sunflower 4	0,9991	-3,479	12,01	12,13	0,13	Ok
sunflower 5	0,9985	-3,451	13,09	12,96	0,14	Ok
sunflower 6	0,9997	-3,429	11,96	12,08	0,11	Ok
buckwheat 1	0,9939	-3,298	9,90	9,72	0,18	Ok
buckwheat 2	0,9993	-3,420	9,89	9,80	0,09	Ok
buckwheat 3	0,9996	-3,408	11,31	11,23	0,08	Ok

Mean Cq values of undiluted and serial diluted DNA extracts

Sample code	Working dilution	Diluted samples			
	1:1	1:4	1:16	1:64	1:256
sunflower 1	12,98	14,99	16,98	18,99	21,06
sunflower 2	15,77	18,08	20,04	22,17	24,40
sunflower 3	14,35	16,50	18,64	20,22	22,68
sunflower 4	12,13	14,14	16,19	18,19	20,45
sunflower 5	12,96	15,18	17,30	19,20	21,47
sunflower 6	12,08	14,05	16,08	18,14	20,24
buckwheat 1	9,72	11,80	13,89	16,09	17,69
buckwheat 2	9,80	11,92	14,03	16,13	18,09
buckwheat 3	11,23	13,38	15,40	17,44	19,54

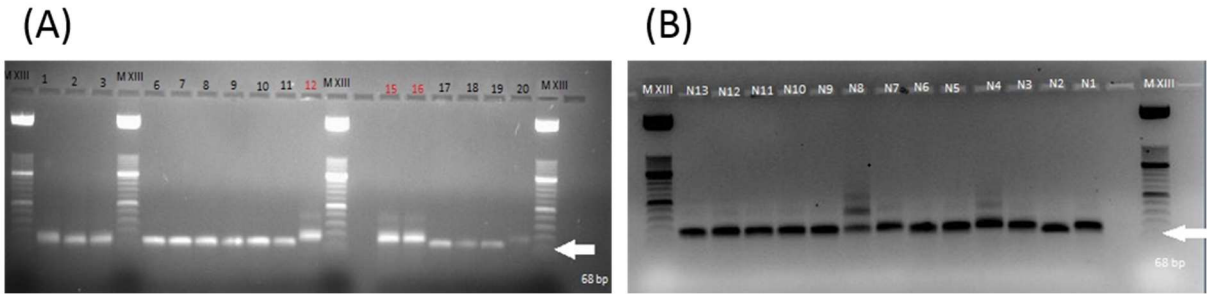
An 18S rDNA region was amplified according to Christensen et al. (2004) All extracts analysed show no inhibition according to ENGL criteria. Thus, extracts can be further used for qualitative and quantitative PCR analyses although fluorometrically measured DNA quantity is very low.

5. Determination of amplifiability by conventional and dPCR

Amplifiability of DNA extracts were assessed by conventional PCR and agarose gel electrophoresis (

Figure A1.4).

Figure A1.4. Agarose gels from honeybee product extracts after 18S rDNA PCR. (A) Sample 1-3: buckwheat pollen, Sample 6-11: sunflower pollen, Sample 14-19: dandelion pollen (B) Unifloral honey (N1 – N13)



Bands at position 68 bp show that PCR amplification is successful. The minor differences in length may result from different pollen genotypes.

To further test amplifiability and determine whether absolute copy numbers are sufficient to address regulatory requirements for GMO-analysis, absolute copy numbers (18S rDNA) were determined by dPCR (Table A1.1).

Table A1.1. PCR with 18S rDNA-primer according to protocol (*information available on request)

Matrix (common name)	latin name	Average copy number per µl	Dilution factor for PCR
Black locust pollen	*Robinia pseudoacacia*	56	10,000
Limeseed pollen	*Tilia spp.*	28	10,000
Rapeseed pollen	*Brassica napus*	34	10,000
Sweet chestnut	*Castanea sativa*	161	10,000
Beebread		623	10,000
Propolis		6,573	100
Royal jelly		6	1,000
Monofloral honey (average copy number of N1-N13)		13	10,000

The LOD of the ddPCR method is between 25-60 copies/µl. It is common that LOD of GMO-events is around 5-10 copies. It is shown here that this extraction method has a sufficient DNA-yield (enough copies) to be above the common LOD. Thus, all extracts could be detected reliably. For selected extracts, the dilution factor should be adapted for further analyses (e.g. lime, royal jelly, monofloral honey).

6. Summary and Conclusion

Results show that the extraction method of choice is suitable to extract pure and amplifiable DNA from pollen from different flowering plants. Moreover, it can be used to extract DNA from food products such as e.g. propolis, royal jelly or beebread.

➔ **Method successfully validated for honeybee products.**

Annex II. Example workflow for inter-laboratory validation of matrix-specific extraction method

To plan and implement an inter-laboratory validation study several aspects have to be taken into consideration. First, during intra-laboratory validation (ideally performed by more than one single laboratory) the DNA extraction method of interest should have shown its applicability for the intended use.

DNA has to be extracted from a representative number of test materials. For universal extraction methods, this means that at least five different sample matrices, with one sample material per matrix should be used. For matrix-specific extraction methods at least three sample materials from a defined type of matrix should be tested.

According to IUPAC recommendations (Horwitz, 1995), data of at least eight participating laboratories should be available for data evaluation. If acquisition of eight participating laboratories is, due to specific responsibilities or needs, not possible the number of participants could be reduced to five. According to this, the laboratory/institution that organizes the inter-laboratory validation has to prepare sufficient sample material to

(a) assess sample quality adequately (extractability, homogeneity and stability)

(b) hand out enough sample material to all (at least eight or five respectively) participating laboratories.

Preparation of samples can be done in collaboration with a service contractor with accreditation under ISO/IEC 17025:2017.

To ensure, that participants follow a distinct experimental procedure, handing out a precise protocol mentioning the aim of the validation study, detailed description of experimental procedure, minimal requirements according to technical equipment, personnel and time resources is of major importance. This information should be sent first, before the sample material is handed out (e.g. when laboratories are invited).

After the experimental phase, relevant data should be submitted in a way that makes further processing and evaluation possible. For inter-laboratory validation, studies performed with the same criteria as those defined for in-house validations are evaluated. On top a summary that includes results of all laboratories should be included.

Performance requirements for DNA extraction methods that are used to prepare DNA extracts for distinct PCR methods are:

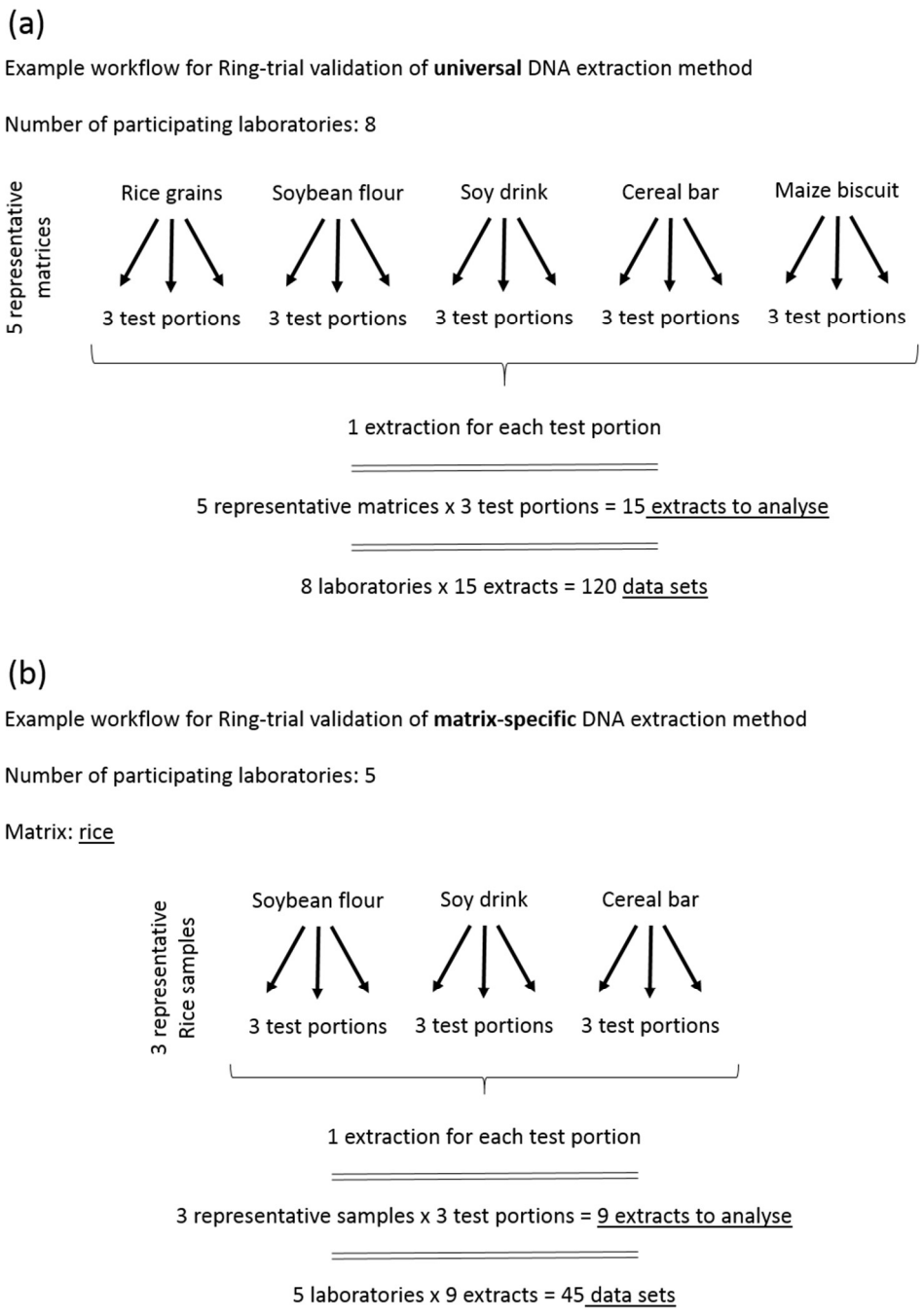
- At least 1,000 amplifiable copies of a reference gene should be present.
- All DNA extracts generated from test samples should give positive PCR results.
- None of the extraction replicates should be affected by PCR inhibition.

Using real life samples, especially highly processed food and feed material, achieving these requirements can be very challenging. Therefore, if initial results indicate that only minor amounts of amplifiable DNA can be extracted from test samples (e.g. for highly processed matrices) performance requirements should be attenuated. In this case:

- In 90% of DNA extracts, 1,000 amplifiable copies of a reference gene should be present.
- 90% of all DNA extracts should give positive PCR results.
- At least 80% of all DNA extracts should not be affected by PCR inhibition.

An example of a ring-trial validation is shown in Figure A2.1.

Figure A2.1. Example workflow for Ring-trial validation according to Waiblinger et al. (2014) (a) Workflow for a universal DNA extraction method and (b) Workflow for a matrix-specific DNA extraction method.



Annex III. EU Survey on DNA extraction from food and feed 2017

Survey organised by the EURL GMM (W. Broothaerts, M. Burns and J. Žel). Answers received from 38 laboratories (NRLs and OCLs).

Results presented during the training workshop on DNA extraction organised by the EURL GMFF in Ispra, IT on 7-9 June 2017, with support of LGC, UK.

1. Which type of samples are being analysed in your lab for qPCR applications (i.e., requiring DNA extraction)?

		Answers	Ratio
Maize seed/grain		38	100%
Soybean seed (raw beans)		37	97.37%
Oilseed rape seed		31	81.58%
Rice seed/grain		31	81.58%
Cotton seed		12	31.58%
Potato		16	42.11%
Sugar beet		14	36.84%
Vegetable seed		12	31.58%
Animal feed from plant origin (compound seeds)		28	73.68%
Animal feed from non-plant origin (meat or fish based)		12	31.58%
Vegetables and fruits (leaves, stems, flowers, roots, but not seeds)		19	50%
Processed food products rich in carbohydrates (bread, pizza, biscuits, jams, ...)		28	73.68%
Processed food products rich in fat (cheese, butter, oil, nuts, ...)		23	60.53%
Processed food products rich in proteins (yoghurt, [soya] milk, meat, fish, ...)		27	71.05%
Highly processed food (corn gluten, soy lecithin, ...)		24	63.16%
Chocolate		12	31.58%
Honey		12	31.58%
Feed additives (e.g. choline chloride)		12	31.58%
Other, please specify below		3	7.89%
No Answer		0	0%

2. Which DNA extraction methods are being used in your lab (please tick all that are being used)?

		Answers	Ratio
CTAB (more details to be provided below)		31	81.58%
NucleoSpin Food		15	39.47%
NucleoSpin Plant		6	15.79%
GeneSpin		2	5.26%
Promega Wizard		8	21.05%
DNeasy Plant		10	26.32%
DNeasy Mericon Food		7	18.42%
Biotecon Foodproof		3	7.89%
Maxwell 16 Food, Feed, Seed		2	5.26%
SDS		6	15.79%
Other, please specify below.		11	28.95%
No Answer		0	0%

Other: SureFood prep (advanced), Fast ID genomic DNA extraction kit, KingFisher + magnetic beads kit MN, Generon Ion Force, DNeasy Bood and Tissue kit, CTAB + NucleoMag Plant kit

3. Which additional DNA purification methods are being used in your laboratory following the DNA extraction method?

		Answers	Ratio
No additional clean-up		23	60.53%
Additional ethanol precipitation		5	13.16%
Eurofins DNAExtractor cleaning column		1	2.63%
Promega Wizard DNA clean-up resin		7	18.42%
Qiagen QIAQuick		8	21.05%
Qiagen Genomic-Tip 20/G		2	5.26%
Polyethylene glycol (PEG)		0	0%
Other, please specify below		5	13.16%
No Answer		0	0%

Other: QIAEX II, Genomic DNA clean-up MN, Genomed JETQuick PCR product purification spin kit Imtec.

4. Which modifications to standard DNA extraction methods are you applying (always or for specific matrices)?

Increased sample intake
Larger volume of lysis buffer
Increased incubation time
Addition of RNase, Proteinase K- or α-amylase
Longer centrifugation time, increased speed
Defatting with n-hexane
Additional ethanol (+ K-acetate) precipitation
Other matrix-dependent modifications, e.g. for OSR Dellaporta buffer+Qiagen DNeasy, CTAB modifications depending on matrix

5. Do you use a structured decision tree in your lab to choose the extraction method for a given sample matrix?

		Answers	Ratio
Yes		13	34.21%
No		25	65.79%
No Answer		0	0%

6a. DNA extraction method(s) used for <u>seed matrices</u> (+ tubers):

	Maize	Soybean	Oilseed rape	Rice	Cotton	Vegetable seed
CTAB	22	21	15	15	7	7
NucleoSpin food	8	9	8	6	3	2
NucleoSpin plant	3	4	4	1	2	1
GeneSpin	0	1	0	0	0	0
Wizard	3	5	3	3	1	1
DNeasy plant	2	4	3	1	1	2
Mericon food	2	4	3	3	1	2
Biotecon	1	1	1	1	0	0
Maxwell food	0	2	2	0	0	0
SDS	4	5	3	3	1	2
Other	6	7	5	4	4	1
No Answer	0	1	8	7	24	24

6b. DNA extraction method(s) used for feed matrices:

	Feed plant-based	Feed animal-based	Feed additives
CTAB	17	8	8
NucleoSpin food	6	5	4
NucleoSpin plant	1	1	1
GeneSpin	1	0	1
Wizard	3	2	1
DNeasy plant	1	0	1
Mericon food	1	0	0
Biotecon	1	0	0
Maxwell food	0	0	0
SDS	2	1	1
Other	4	1	1
No Answer	11	25	26

6c. DNA extraction method(s) used for vegetables/fruits/tubers:

	Vegetables/fruits	Potato tubers	Sugar beet roots
CTAB	12	6	5
NucleoSpin food	6	3	3
NucleoSpin plant	1	1	2
GeneSpin	0	0	0
Wizard	1	2	1
DNeasy plant	2	3	1
Mericon food	1	0	1
Biotecon	1	0	0
Maxwell food	0	0	0
SDS	0	1	1
Other	3	1	1
No Answer	18	23	25

6d. DNA extraction method(s) used for food matrices:

	Processed food, carbohydrate-rich	Processed food, fat-rich	Processed food, protein-rich	Highly processed food (gluten, lecithin)	Chocolate	Honey
CTAB	17	16	15	14	7	5
NucleoSpin food	11	8	11	6	4	4
NucleoSpin plant	0	0	0	0	1	0
GeneSpin	1	1	1	1	1	0
Wizard	3	3	3	0	0	1
DNeasy plant	0	0	0	0	0	0
Mericon food	5	4	4	2	0	0
Biotecon	2	1	1	2	2	0
Maxwell food	1	0	0	0	0	0
SDS	3	3	3	1	0	1
Other	4	4	4	7	3	4
No Answer	10	14	12	16	28	26

Comments on special matrices:

- Cotton seed (only during ISTA PT, not yet used in routine): CTAB/Genomic-tip 20 (CRLVL13/04XP); Lecithin (as well as vegetable oil): Würz et al. (1998) (not applied on gluten-based samples)
- For DNA extraction from lecithin we used n-hexane; guanidine thiocyanate buffer

- NUCLEOSPIN KIT: in case of highly processed food, like corn flakes and nachos, the starting material amount could be increased (up to 3-4 times). NucleoSpin Food does not work for starch and performs poorly for rice matrix. For starch a CTAB based method is used
- Maize glutens are extracted with the MN Nucleospin Plant II Kit including a Proteinase K digestion for 1 h.
- In-house CTAB approach appears to be generally applicable to a wide range of matrices. Whilst time consuming, it can be adapted as necessary for individual matrices.

7. Which method do you use to determine the DNA quantity obtained?

		Answers	Ratio
Generally not determined		3	7.89%
Spectrophotometer (OD)		33	86.84%
Fluorimeter (e.g. PicoGreen)		5	13.16%
qPCR (e.g. for endogenous reference gene)		17	44.74%
Other, please specify		1	2.63%
No Answer		0	0%

Other: Horizontal agarose electrophoresis, dPCR

8. Select the approach used to show absence of PCR inhibition.

		Answers	Ratio
None (no inhibition is suspected based on experience)		1	2.63%
We check that the optical density ratios (OD260/280, 260/230) are acceptable		23	60.53%
We verify that the amplification curves look normal		18	47.37%
We run two dilutions and verify if the delta Cq or GM % are as expected		18	47.37%
We run three or four dilutions and verify if the delta Cq or GM% are as expected		10	26.32%
We perform a PCR inhibition run with a reference gene before analysis: 3 or 4 dilutions, linear regression, extrapolation of Cq for undiluted extract, compare this to the measured Cq		9	23.68%
We add an internal positive control to the reactions and check the Cq		7	18.42%
Other, please specify below.		2	5.26%
No Answer		0	0%

Other: Qualitative purpose (conventional PCR assay targeting "universal plant" *rbcL* gene, just to check if there is DNA that can be amplified by PCR, PCR on two dilutions) ;
Quantitative purpose : PCR on two dilutions + following rules from ISO 21570:2005 (section 7.5)

9. Do you check the DNA quality by gel electrophoresis?

		Answers	Ratio
Always		3	7.89%
Regularly		0	0%
Only for new matrices extracted		4	10.53%
Sometimes		9	23.68%
Never		22	57.89%

10. Please clarify the composition of the CTAB extraction buffer (tick also those added separately or subsequently before organic extraction).

		Answers	Ratio
CTAB 2%		30	78.95%
Tris-HCl		30	78.95%
EDTA		29	76.32%
1.4-1.5 M NaCl		28	73.68%
RNase A		24	63.16%
Proteinase K		28	73.68%
ß-mercaptoethanol		1	2.63%
Polyvinyl pyrrolidone (PVP)		0	0%
Other, please specify below		3	7.89%
No Answer		7	18.42%

Other: Alpha-amylase for carbohydrate rich matrices

11. Which kind of organic extraction do you perform?

		Answers	Ratio
No organic extraction		3	7.89%
Phenol/chloroform (+/- isoamyl alcohol or octanol)		4	10.53%
Chloroform (+/- isoamyl alcohol or octanol)		25	65.79%
No Answer		7	18.42%

12. Immediately following (phenol) chloroform extraction, how is the DNA precipitated out of the aqueous phase?

		Answers	Ratio
CTAB precipitation (CTAB+NaCl)		17	44.74%
Isopropanol precipitation (+/- salt addition)		20	52.63%
Ethanol precipitation (+/- salt addition)		5	13.16%
No Answer		7	18.42%

Annex IV. Devices used for DNA concentration / purity measurements in pre-PCR check

1. Spectrophotometry

Widely used NanoDrop® (Thermofisher Scientific) or NanoVue™ (GE Healthcare) spectrophotometers were designed and manufactured to measure the absorbance of small volume samples of nucleic acids and proteins without the use of cuvettes or capillaries.

NanoDrop™ 1000 (Thermofisher Scientific) Spectrophotometer measures 1 µl samples and has the capability to measure highly concentrated samples without dilution.

NanoVue Plus Spectrophotometer (GE Healthcare) is a stand-alone instrument that can be used for quantification of nucleic acids and protein samples. The instrument utilizes 0.5 to 5 µl of the sample put on a sample plate. It is pre-programmed with a range of pre-set methods for the quantification of nucleic acids and proteins, using UV or dye-intercalation methods (Lowry, Bradford, BCA, and Biuret). The instrument does not require a computer because it has a direct user interface.

The FLUOstar® Omega is a multi-mode microplate reader with six detection modes. It utilizes an ultra-fast UV/vis spectrometer or filters for absorbance as well as highly sensitive filters for all other detection modes. Absorbance was measured at 260 nm and 280 nm on an FLUOstar Omega plate reader. The absorbance reads were performed three times.

An advantage of this method is that the volume of DNA used for quantification can be returned to the original sample post-analysis. A comparison of DNA quantification values obtained by UV spectrophotometry (including NanoDrop and FLUOstar) and PicoGreen analysis: DNA concentration values determined by UV spectrophotometry were significantly higher than those determined by PicoGreen®. The consistently higher values for the UV spectrophotometry methods are attributable to the inability of UV spectrophotometry to distinguish between double-stranded and single stranded nucleic acids. The values obtained using the NanoDrop were consistently higher than those obtained using the FLUOstar Omega. In conclusion, PicoGreen values are more reliable for downstream applications, but UV analysis has to be done to define the purity of DNA (LGC, technical note).

Some instruments (e.g. NanoDrop One) may in addition to the accurate quantification of nucleic acids (and protein) samples also provide information about sample quality by identifying common contaminants (protein, phenol, guanidine HCl, guanidinium isocyanate).

2. Fluorimetry

The QuantiFluor® dsDNA System (Promega) contains a fluorescent DNA-binding dye that enables sensitive quantification of small amounts of dsDNA in a purified sample. The assay is highly selective for dsDNA over other nucleic acids and is linear over a range of 0.05–200 ng of dsDNA input (0.05–200 ng/µl from 1 µl of original sample).

The Qubit fluorometer (ThermoFisher) is a DNA quantification device based on the fluorescence intensity of fluorescent dye binding to dsDNA using the Qubit™ assays that contain advanced dyes specific for DNA, RNA, or proteins. Detection technologies used in the Qubit Fluorometer help attaining the highest sensitivity using 1 µl of sample still achieving high levels of accuracy, even in the case of very diluted samples (Schipor et al., 2016; Qubit 2 manual, 2010). With two different assays it is possible quantify dsDNA concentration in a range of 0.01–1000 ng/µl.

3. Capillary electrophoresis

The QIAxcel Advanced is a CE device that includes an array of light-emitting diodes and micro-optical collectors that latch to capillaries within QIAxcel gel cartridges. Fragments that are migrating through a gel matrix within the capillary pass excitation and detection spots and the signal is transmitted through a photomultiplier tube to the QIAxcel ScreenGel software for data interpretation.

The high detection sensitivity provided by the QIAxcel Advanced instrument (Qiagen) enables robust results even with low concentrations of nucleic acid. With a resolution of 3–5 bp for fragments smaller than 0.5 kb, the QIAxcel Advanced system ensures greater accuracy than slab-gel methods, as well as greater confidence in data interpretation. Sample consumption is less than 0.1 µl per analysis. Hands-free sample loading and self-contained components minimize exposure to hazardous chemicals, such as ethidium bromide. Other devices

e.g. are Bio-Rad BioFocus 2000 and BioFocus 3000 system, Agilent 7100 CE System, Promega Spectrum Compact CE System.

4. On chip technologies

The 2100 Bioanalyzer system and 4200 TapeStation are chip-based nucleic acid analysis system enables rapid, accurate and reproducible analysis of the integrity and quality of RNA, DNA and protein samples and an estimation of sample concentration, using small volume sample. It is the recommended method for quality control of RNA and/or DNA prior to downstream analysis like quantitative Real Time PCR, microarray, reverse transcription, next generation sequencing, etc. (Agilent).

Annex V. Examples of DNA extractions from challenging samples

1. Maize gluten

Highly processed protein-rich feed components, like maize gluten, contain small amounts of intact DNA suitable for PCR analysis and many PCR inhibitors, therefore an extraction method that yields a sufficient amount and quality of DNA is crucial. To establish a suitable method for extraction of DNA from maize gluten the Working Group of the VDLUFA (Association of German Agricultural Analytic and Research Institutes) conducted two inter-laboratory comparison tests involving different DNA extraction methods (Matthes et al., 2020). Of all evaluated methods the described CTAB protocol yielded sufficient amounts of amplifiable DNA to control the compliance with the permissible limits and labelling thresholds of authorised GM maize events. The extraction protocol with Wizard DNA Clean-up System kit (Promega) using SDS lysis buffer had better reproducibility but on average five times lower yield and quality of amplifiable DNA than with the CTAB protocol. The evaluation was based on the calculated number of amplifiable DNA copies and on the PCR efficiency determined on the measured Cq values of maize specific *HmgA* gene using several dilution steps.

NIB (Slovenia) is using a modified CTAB protocol from Annex A.3 (ISO 21571:2005): 2 g of ground sample is mixed with 5 ml water (Sigma W4502) and 10 ml CTAB lysis buffer (100 mM Tris/HCl; 20 mM Na_2EDTA; 1.4 M NaCl; 2% (w/v) CTAB; pH 8.0) supplemented with 2 mg Proteinase K. The mixture is incubated for 30-90 min at 65 °C under agitation. One mg RNase A is added and incubated for 5-10 min at 65 °C. Following centrifugation for 20 min at 5000 x g, 10-15 ml chloroform is added to the supernatant and mixed for 30 s. The chloroform step is repeated. DNA is precipitated from the upper aqueous phase by the addition of two volumes of CTAB precipitation solution (40 mM NaCl, 0.5 % CTAB; pH 8.0), followed by an incubation step of 60 min at room temperature and pelleted by centrifugation for 20 min at 5000 x g. The pellet is resuspended in 700 µl 1.2 M NaCl and extracted with 700 µl chloroform. Phases are separated by centrifugation and the DNA from the aqueous layer is precipitated by addition of 0.6 volumes of isopropanol, an optional incubation step at -20 °C, and sedimented by centrifugation for 10 min at 18.000 x g. The supernatant is carefully discarded. The pellet is washed with 500 µl 70% (v/v) ethanol, centrifugated again for 10 min and the supernatant discarded again. The DNA is dried for several minutes at 50 °C and dissolved in 150 µl water (Sigma W4502).

2. Lecithin and chocolate

In most cases, the only soy derived ingredient used in chocolate manufacturing is soy lecithin. Complex matrices such as chocolate contain a number of PCR inhibitors, e.g. polysaccharides and polyphenols, which may impede the amplification of the extracted DNA. Moreover, in the case of chocolate, difficulty in amplification of the lectin gene can be caused by the low concentrations of soy lecithin present in products of the sweetener industry. Therefore, an efficient extraction procedure is of the highest importance. Many factors however, such as the choice of extraction method, sample size and PCR protocol influence the PCR end result. Therefore, preconditions regarding the quality, quantity and purity of the extracted DNA should be carefully determined to guarantee a successful GMO analysis (Gryson et al., 2004, 2007).

An optimised protocol for the extraction of soybean DNA developed by Würz et al. (1998) is still used by many laboratories for DNA extraction from chocolate and lecithin.

In Gryson et al. (2004) five different methods for the extraction of DNA from chocolate were evaluated, using four commercially available extraction kits: (DNA Stool Mini (Qiagen), Nucleon PhytoPure (Amersham), Wizard Magnetic DNA Purification System for Food (Promega), Genespin (Genescan) and a non-commercial CTAB method proposed by CEN (ISO 21571). DNA quality and quantity were evaluated with gel electrophoresis of the PCR amplified soybean-specific lectin gene. The performance of CTAB-based method was the best, taking into consideration the adaptations of the extraction procedure, although this method was more time-consuming than the others.

Ring trial validation of a method for DNA extraction from soy lecithins was published by Waiblinger et al. (2007). The protocol with slight modifications within the recipe of the extraction buffer is described in the Swiss food manual (SLMB, 2004). Five different samples of soybean lecithin were tested. Amplification of the soybean lectin gene with real-time PCR was used for assessment of DNA extraction. In four out of five samples, mean practical LOD values below 0.9% were obtained.

3. Pollen and honey

The 2011 the EU Court of Justice ruling highlighted the need for validated methods for extraction of DNA from pollen in honey. In response to this the EURL GMFF shortly released a SOP describing a CTAB-based extraction

method (EURL GMFF, 2012). The method is intended to extract genomic DNA from maize, soya and rapeseed pollen present in honey and uses a filter to remove the pollen, which is then disrupted, and the DNA extracted using a CTAB approach.

Mainly all published DNA extraction protocols from honey and bee pollen are using beads for mechanical disruption and filters for pollen fraction enrichment.

DNeasy Blood and Tissue Kit (Qiagen) was used for DNA extraction from honey samples after pre-treatment with beads and digestion with Proteinase K (Laube et al., 2010).

The method described by BVL (2011) is a CTAB-based method referring to the protocol published in Waiblinger et al. (1999, 2005).

In Waiblinger et al. (2012b) an in-house and inter-laboratory validation with a further optimised extraction method from pollen and honey is described according to the following protocols: Waiblinger et al. (1999, 2005) and BVL (2011). The method is CTAB-based with subsequent clean–up with QIAQuick PCR purification Kit (Qiagen).

Guertler et al. (2014) published an automated DNA extraction method from pollen in honey using the Maxwell 16 instrument (Promega) and the Maxwell 16 FFS Nucleic Acid Extraction System (Promega) Kit. The automated method was compared with a manual CTAB-based method based on Waiblinger et al. (2012b). The automated DNA extraction was faster and resulted in a higher DNA quantity and sufficient DNA purity. Real-time PCR results obtained after automated DNA extraction are comparable to results after manual DNA extraction. No PCR inhibition was observed. The applicability of this method was further successfully confirmed by analysis of different routine honey samples.

Soares et al. (2015) compared 15 extraction methods (Macherey-Nagel NucleoSpin Plant A and B, Qiagen DNeasy Plant Mini Kit, CTAB-based and Wizard methods combined with three different pre-treatments) in terms of DNA integrity, yield and purity for four honey sample. The Wizard method demonstrated the best efficacy in terms of DNA quality and amplification capacity assessed by electrophoresis and conventional PCR.

An in-house validation of DNA extraction protocols from honey and bee pollen is described in Torricelli et al. (2016). Four extraction protocols were combined and tested (combination of Laube et al. (2010) and BVL (2011) pre-treatments and DNA extractions). Cq and ΔCq were considered to assess DNA quantity and quality, respectively, in further PCR analysis as the most reliable approach. In conclusion, the best extraction method was derived from a combination of a pre-treatment (BVL, 2011) and BVL (2011) extraction method, with improvements by the addition of RNase A, by removing the isopropanol and 70% EtOH steps and by the purification with NucleoSpin gDNA clean-up.

For reference DNA detection, actin and tRNA-Leu plant-specific genes are used and evaluated by Laube et al. (2010).

LGC (UK) used the published EURL GMFF (2012) method to demonstrate that amplifiable DNA could be extracted from pollen across a range of commercially sourced honey products, with no or only minor modifications to the protocol. The method was used to successfully extract DNA from samples of five different honey products labelled as English Country Honey, Pure Set Honey, Organic Honey, Acacia Honey, and Wildflower Honey. A universal PCR assay (internal transcribed spacer 2 region of nuclear ribosomal DNA) was applied to the extracted DNA and PCR products analysed using capillary electrophoresis to confirm successful amplification. The study demonstrated that amplifiable DNA can be successfully extracted from different honey products using the EURL GMFF SOP, inclusive of minor variations to the protocol. Minor modifications to the EURL GMFF protocol which still produced amplifiable DNA from pollen from honey products included the use of a specific single temperature for dissolving the honey (50 °C), and the use of a syringe filter with 10 µm filter membrane instead of the vacuum filter and 12 µm filter membrane described in the original SOP.

AGES (Austria) performed a project on bee products to find one optimised successful DNA extraction method for all relevant matrices (honey, pollen, propolis, beebread, gelee royal). Prior to lysis a washing and defatting step followed by dehydration over night was applied. A modified working buffer including Sarcosyl (N-lauroylsarcosin), PVP10 and sodium disulfite as well as Proteinase K was then used for lysis. Further treatment was performed according to the conventional CTAB-protocol. The yield resulted in 50 % more DNA-copies/µl compared to manual CTAB-protocols, which was demonstrated fluorometrically and additionally with dPCR (AGES, unpublished data). In routine analysis honey samples are usually extracted with a CTAB-protocol from Promega using the Maxwell RSC instrument due to an efficient turnover rate of samples (Maxwell 16 FFS Nucleic Acid Extraction System Kit, Promega).

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