Chapter 15
Nucleic Acid Sample Preparation for Quantitative and Qualitative GMO Analysis

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Abstract According to ISAAA (International Service for the Acquisition of Agri-biotech Applications), the acreages of GMO (Genetically Modified Organism) cultivation worldwide increased in 2012 by 3 % over 2013. The acceptance of genetically modified foods by consumers is still disputed, and concerns about their safety persist in the public opinion. Whatever their opinion, European consumers for example wish to keep their freedom of choice by having a reliable labeling of products containing GMO, above a threshold of fortuitous presence which has been defined in the EU at 0.9 % and different worldwide. GMO analysis includes several steps ranging from sampling, sample preparation, choice of fit for purpose analytical method(s), analytical procedure itself (DNA isolation, screening, and/or GMO identification and quantification), and result interpretation. One of the major steps of the procedure is the nucleic acids preparation because of the complexity of the matrixes analyzed such as food, feed, seeds, grains, or plant species. The aim of this chapter is to give an overview of the existing methods and strategies for nucleic acids sample preparation in the frame of GMO analysis along the procedure of GMO detection and quantification using different matrixes and to highlight the principal gaps and the future needs.

Keywords GMO • Analysis • Isolation • Sampling • PCR • Detection • Quantification

1 Nucleic Acid Isolation Used in GMO Analysis

The consequence of a threshold setting, and the detailed traceability and labeling requirements with different thresholds all over the world, gives rise to the specific need for new analytical methods for the reliable detection, identification, and quantification of a given authorized GM line, in particular, for general enforcement and control activities and in the event of any possible future risk management requirement. In fact, the general procedure for GMO detection and quantification

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analysis is based on sampling and sub-sampling, grinding and homogenization of the sample, analyte extraction (nucleic acids or proteins), and finally the application of the adequate technique and analysis of the results. Nowadays, nucleic acids analyses are considered as an important analytical tool for food analysis, especially, after the approval and the cultivation of various genetically modified crops in the USA and Europe [1]. The aim of a nucleic acid extraction method is to isolate DNA of suitable integrity, purity and of sufficient quantity for diagnostic applications by qualitative or quantitative real-time PCR [2]. Obtaining DNA of high quality is paramount for ensuring confidence in all subsequent steps in the process of generating analytical measurements. This chapter reviews DNA isolation methods that are commonly utilized in GMO analysis and bottlenecks for their use depending on the matrix used [3].

2 Methods Used for DNA Isolation

The first step in the DNA extraction procedure is the preparation of the sample. In the case of a homogeneous sample, such as a commodity crop or a single ingredient food product, the whole sample may be considered to be representative [4]. In this case sampling strategies are applied (From bulk sample to laboratory sample). However, for a heterogeneous composite food product or, particularly when several of the components may contain GM derivatives, e.g., pizza, the issue of sufficient homogenization to ensure a representative sample is particularly critical, especially if a quantitative GM analysis is required [2]. Once a homogeneous and representative sample for a particular batch has been obtained, the analyst can choose among a vast range of methods. This choice is a crucial depending on the composition of the sample and the ingredients. Plenty of methods in GMO analysis are based on precipitation of the DNA using CTAB extraction buffer (cetyltrimethylammonium bromide) (Table 15.1). These methods are considered efficient for a wide range of plant-derived foods, in particular for separation of polysaccharides from DNA [11, 13, 17, 21, 22]. Costs are lower than those for commercial kits because of the use of common chemicals (not taking the labor costs into account) [5, 11, 17]. Other methods used are based on DNA binding to resins (Table 15.2) and magnetic particles (Table 15.3), prepacked glass fibers [16], non-chaotropic solid-phase extraction [13], use of PVPP [5, 26, 27], and the FTA card, which can be immediately used as a PCR template [25]. So far it has been very difficult, or impossible, to obtain good quality DNA for PCR from highly processed food such as cornflakes, corn puffs, hydrolyzed plant proteins (soya sauce), purified lecithin, and starch derivatives (maltodextrins, glucose syrup) because of both PCR inhibitors and very low yields [18, 19, 28–30]. As a result of DNA degradation and the presence of PCR inhibitors, extraction of the DNA from processed foods is often a balance between high yields and high purity. Extraction methods for GM food products have already been compared by several researchers, showing that some extraction methods are better suited to isolation of DNA from processed foodstuffs than others, proving that a

Table 15.1 Resin-binding DNA extraction methods frequently used in GMO analysis

Method	Samples	Other methods[a]	References
Chelex 100	Tofu, soybean flour, lecithin	A, U, C, N	[5]
DNeasy method	Tofu, soybean flour, lecithin	A, U, C, N	[5]
DNeasy plant mini kit	Polenta, crackers, tacos, tofu	A, U, C, R	[6]
	Corn and soya flour, biscuits, chocolate based products,	U, C	[7]
	Miso, taco shells, soya protein	A, F, R	[8]
	Corn flour, corn starch		
DNeasy tissue kit	Flour, feed, oil	C	[9]
DNeasy plant maxi kit	Corn starch	A, F, R	[8]
GenElute plant genomic kit	Corn flour, canned maize, corn puff snacks, corn chip	A, U, N, R	[10]
	Snacks, cornflakes, infant formula		
GeneSpin DNA isolation kit	Biscuits, chocolate A,	C	[11]
	Soybean, soybean flour, soybean drinks, protein isolates, soybean sauce, tofu, soybean dessert, vegetarian soy products	U, F, C, R	[12]
		U, F, C	[13]
	Flours, biscuits, instant paps		
Method A, B	Maize kernels	U, C, R	[14]
NucleoSpin food kit	Miso	U	[15]
	Polenta, crackers, tacos, tofu	A, U, C, R	[6]
	Corn flour, corn starch	A, F, R	[8]
	Soybean, soybean flour, soybean drinks, protein isolates, soybean sauce, tofu, soybean dessert, vegetarian soy products	U, F, C, R	[12]
	Miso	U	[15]
	Soy flour, polenta, soymilk, soy bread, maize bread, fresella, cracker, chocolate snack	U, C	[16]
Nucleon PhytoPure kit	Biscuits, chocolate	A, C	[17]
	Tofu, soybean flour, lecithin	A, U, C, N	[5]
Plant genomic DNA	Miso	A, F, R	[15]
extraction miniprep			
system			
QIAamp DNA stool	Biscuits, chocolate	A, C	[11]

(continued)

Table 15.1 (continued)

Method	Samples	Other methods[a]	References
mini kit	Corn flour, canned maize, corn puff snacks, corn chip snacks, cornflakes, infant formula	A, U, N, R	[10]
	Corn and soya flour, biscuits, chocolate based products, miso, taco shells, soya protein	U, C	[7]
	Soybean flour, polenta, soymilk, soy bread, maize bread, fresella, cracker, chocolate snack	U, C	[16]
		A, U, C, R	[6]
	Polenta, crackers, tacos, tofu		
Wizard method	Maize flour, polenta	A, U, N, R	[10]
	Tofu, soybean flour, lecithin	A, U, N	[5]
	Polenta, crackers, tacos, tofu	A, U, C, R	[6]
	Canned maize, corn snacks, cornflakes, infant formula	A, U, N, R	[10]
	Various foodstuffs	U, C	[18]
	Soybean flour, soybean proteins	C, N	[19]
	Corn flour, canned maize, corn puff snacks, corn chipsnacks, cornflakes, infant formula	A, U, N, R	[10]
	Various foodstuffs	C, R	[20]

Springer-Verlag, Anal Bioanal Chem, Effect of food processing on plant DNA degradation and PCR-based GMO analysis: a review, 396(6), 2009, pp 2003–2022, Gryson N.
aA agarose gel electrophoresis, *F* fluorescence measurement, *C* conventional PCR, *N* nested PCR, *R* real-time PCR, *U* UV spectrophotometry

particular method should be chosen on a case-by-case basis. Tables 15.1, 15.2, and 15.3 summarize different studies in which DNA extraction methods are compared, using agarose gel electrophoresis (A), UV spectrophotometry (U), fluorescence measurements (F), conventional PCR (C), nested PCR (N), or real-time PCR in their evaluation.

The type of extraction can significantly affect the measurement results of a PCR assay [6, 8, 31–34]. Moreover, fractions of different particle size distribution may lead to unequal extraction efficiencies, which may lead to bias in GMO analysis results [35–39]. Because of the wide variety of extraction methods present on the market and/or adaptation of specific methods to increase DNA yields (e.g., change of sample weight, buffer volumes) comparison between the recoveries of particular methods is nearly impossible. Also, when the DNA yield seems to be too poor for subsequent DNA measurement, the extraction is sometimes scaled up [8, 11, 17, 26, 27, 40–43], which might change the overall extraction efficiency. Other factors affecting the extraction efficiency are the presence of chemicals in the sample, for example fungicides [44], physicochemical changes during processing, which lead to binding of DNA to insoluble matrix components [45, 46], oxidation or enzyme hydrolysis of DNA [47], and the length of the DNA to be extracted [8]. Food processes, for example thermal treatment, which lead to a decrease of DNA fragment length, will also result in changed DNA extraction efficiencies [48].

Table 15.2 DNA precipitation methods frequently used in GMO analysis

Method	Description	Samples	Other methods[a]	References
AbiPrism 6100PrepStation	Manufacturer's manual	45 samples, including soya milk, wafer, soya meat, ice cream, soya cream, cracker, soya lecithin, biscuits,soya drink, soya oil, rice and rice wafers, cornflakes	R	[23]
CTAB extraction	ISO 21571 (2005)	Biscuit, chocolate	A, C	[17], [24]
	(Doyle JJ et al. 1990)	Soybean flour, soymilk, infant formula, beverage	A, U, C, N	[24]
	(Lipp M et al. 1997)	Soybean flour, soymilk, infant formula, beverage	A, U, C, N	[15]
	(Lipp M et al. 1997)	Miso	U	[23]
	(Lipp M et al. 1997)	45 samples	R	[8]
	(Lipp M et al. 1997)	Corn flour, corn starch	A, F, R	[10]
	(Lipp M et al. 1997)	Corn flour, canned maize, corn puff snacks, corn chip snacks, cornflakes, infant formula	A, U, N, R	[12]
	(Dellaporta SL et al.1983)	Soybean, soybean flour, soybean drinks, protein isolates, soybean sauce, tofu, soybean dessert, vegetarian soy products	U, F, C, R	[15]
	(Di Bernardo G et al. 2005)	Miso	U	[16]
	Majchrzyk P (2002)	Soybean flour, polenta, soymilk, soybread, maize bread, fresella, cracker, chocolate snack	U, C	[14]
	(Stewart CN Jr et al. 1993)	Maize kernels	U, C, R	[15]
	Joint Research Centre (2002)	Miso	U	[13]
	(Yamaguchi H et al. 2000)	Flour, biscuits, instant paps	U, F, C	[25]
	Not specified	Natto, soy sauce	U, C	[14]
	Manufacturer's manual (Di Bernardo G et al. 2007)	Maize kernels	U, C, R	[16]
		Soybean flour, polenta, soymilk, soy bread, maize bread, fresella, cracker, chocolate snack		

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[a]*A*, agarose gel electrophoresis; F, fluorescence measurement; C, conventional PCR; N, nested PCR; R, real-time PCR; U, UV spectrophotometry

Table 15.3 DNA extraction methods based on magnetic particles used in GMO analysis

Methods	Samples	Other methods[a]	References
Method C	Maize kernels	U, C, R	[14]
Wizard Magnetic + Kingfisher	Corn flour, corn starch	A, F, R	[8]
	Corn flour, corn starch	A, F, R	[8]
Wizard Magnetic DNA purification for food	Corn flour, canned maize, corn puff snacks, corn chip snacks, cornflakes, infant formula	A, U, N, R	[10]
	Soybean, soybean flour, soybean drinks, protein isolates, soybean sauce, tofu, soybean dessert, vegetarian soy products	U, F, C, R	[12]
	Feed, maize flour, maize oil	C	[9]
	Biscuit, chocolate	A, C	[11]

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[a]*A* agarose gel electrophoresis, *F* fluorescence measurement, *C* conventional PCR, *N* nested PCR, *R* real-time PCR, *U* UV spectrophotometry

3 DNA Preparation and International Standardized Protocols

The international Standard provides general requirements and specific methods for DNA extraction/purification and quantification in GMO analysis. The search for genetically modified origin of ingredients is performed by means of the following successive (or simultaneous) steps explained in the ISO21571. After sample collection, nucleic acids are extracted from the test portion. Extracted nucleic acids can be further purified, simultaneously or after the extraction process. Afterwards, they are quantified (if necessary), diluted (if necessary) and subjected to analytical procedures (such as PCR). The ISO 21571:2005 International Standards have been established for food matrices, but could also be applicable to other matrices, such as grains and feed. It has been designed as an integral part of nucleic-acid-based analytical methods, in particular ISO 21569 on qualitative analytical methods, and ISO 21570 on quantitative analytical methods. Preparation of PCR-quality DNA using phenol–chloroform, guanidinium–chloroform, PVP, and CTAB based DNA extraction methods are detailed in the ISO21571 and also Methods for the quantification of the extracted DNA. In addition most of the methods used for DNA extraction in GMO analysis were validated for example in the European Union through the Joint Research Center, Institute for Health and Consumer Protection for GM food and Feed, Biotechnology and GMO unit (http://gmo-crl.jrc.ec.europa.eu/) and validated in interlaboratory tests (Community Reference laboratories) and the European Network of GMO laboratories (ENGL). For this, to ensure the homogeneity of results using the same validated methods in routine analysis in all the ENGL laboratories (http://gmo-crl.jrc.ec.europa.eu/engl/ENGL.html). In the next paragraph many studies conducted to analyze the influence of DNA extraction methods in GMO analysis.

4 Influence of DNA Extraction Method in GMO Analysis

The question to be asked when preparing a nucleic acid sample for GMO analysis is the following: Do you have the same matrix to analyze? The answer is discussed in the paragraph below. In fact, DNA quality is an important parameter for the detection and quantification of genetically modified organisms (GMO's) using the polymerase chain reaction (PCR). Food processing leads to degradation of DNA, which may impair GMO detection and quantification. For this, many authors studied the effect of food procession on plant DNA degradation and then proposed the parameters to be taken into consideration when using processed foods in molecular analysis. Gryson et al. [4], showed that many factors affect the applicability and reliability of DNA-based qualitative and quantitative GMO detection. Food processes involving mechanical stress, high temperature, pH variations, enzymatic activities, and fermentations affect the primary structure of DNA and cause, for example, hydrolysis, oxidation, and deamination of the DNA [49, 50]. Although food processing may lead to increased homogeneity, it will result in significant degradation of DNA or removal of DNA from the sample. This, in turn, will reduce the sensitivity of the analysis and affect limits of detection and quantification [28, 50, 51] which may alter the result of a qualitative [26, 27] and quantitative GMO analysis. For this reasons, targeted regions for the detection and quantification of GMOs are of small sizes such as the PCR based method for the detection of P35S promoter (79 bp) [52]. Moreover, the efficiency of the extraction method will affect the test result, making it impossible to isolate most of the DNA present in the sample and removal of PCR inhibitors. The so-called matrix effects, plant polysaccharides and polyphenolics, feed additives, or reagents used in extraction procedures can be co-purified, which inhibits the PCR reactions [44, 49, 53–56].

In fact, plenty of processing steps affect the state of the DNA present. High temperature and low pH are considered as the major factors which break down DNA. However, in many experiments PCR amplification will remain possible, irrespective of the matrix. The PCR product should, however, be chosen carefully with regard to length and composition, because both might have an effect on degradation of DNA and, therefore, on the GMO quantification result.

Although evaluation of the effect of several conditions on DNA degradation seems to be easy, comparison of results is quite complicated. Not only will the choice of a certain extraction method affect the end result, but also many techniques are used to evaluate DNA degradation. Agarose gel electrophoresis, UV spectrometry, conventional PCR, and real-time PCR and the most widely used in GMO testing, but care should be taken when results are compared. The number of cycles, the amplicon length, and the amount of DNA added to the PCR will affect detection and quantification limits. Preference should be given to short amplicons of 150 bp maximum. When available, internationally validated PCR assays should be preferred. Next to factors related to the PCR assay, some product-related aspects should be taken into account, as PCR inhibitors may also affect the final amplification result. All these factors will affect the final sensitivity of the test. Concurrent evaluation of

the sample by agarose gel electrophoresis and the determination of the DNA content by spectrophotometry or fluorescence measurements will certainly improve assessment of DNA fragmentation experiments and eventual PCR-based GMO analysis. The possibility of using matrix-specific certified reference materials (CRMs) should be further elaborated. Research should be aimed at definition of the ingredients in terms of their DNA quality and PCR amplification ability. Once this has been achieved, these ingredients can be used as CRMs, or they can be used for the production of more complex matrix-specific CRMs. Although it is impossible to produce a CRM for every type of food product, some key products could be selected which represent a specific group of products with great resemblance in terms of composition and processing. Furthermore, studies should include evaluation of the processed products and their unprocessed counterparts in order to evaluate whether changes in GMO quantity are because of DNA degradation or to the bias of the quantification system [57]. This is a huge challenge targeting the preparation of samples in GMO analysis, since the product to be certified is composed finally of different plant species and taxa. In consequence many criteria should be taken into consideration such as the selection of varieties, ploidy and DNA quantity affecting the GMO quantification.

Regarding the impact of various extraction methods, Smith and Maxwell [58] determined the relative concentration of an endogenous corn invertase (*ivr1*) sequence by real-time PCR as a means to compare four different DNA extraction methods with respect to the overall quality and quantity of DNA isolated from lightly processed and severely degraded food products. They suggested that the extraction efficiency was the most important factor influencing amplification of the *ivr1* gene by real-time PCR. Peano et al. [6] have shown that the DNA extraction method had an influence on the "quality" (integrity and purity) and quantity of extracted DNA. Moreover, they investigated the influence of the DNA extraction method on the quantification of corn MON 810 and Roundup Ready soybean CRMs by real-time PCR through comparison of the measured values with those expected. They proposed to use the DNA extraction method that gives the best correlation with the performance of real-time PCR. A recent study called CCQMP60, organized by the Institute of Reference Measurements and Material (IRMM), assessed the impact of the DNA extraction method, the DNA quantity and quality, PCR inhibition, and real-time PCR detection method on the determination of the GM mass fraction of Bt176. It was shown within this study that the quality of extracted genomic DNA was dependent on both the specific procedure performance of each laboratory and the DNA extraction method applied. Moreover, the occurrence of PCR inhibition for less diluted samples was noted, which resulted in an underestimation of the true value for the investigated GM model. Altogether these studies suggested that the DNA extraction method has an influence on the quality and quantity of isolated gDNA amenable to real-time PCR amplification. The interlaboratory comparison employed systematically DNA extraction methods that differ with respect to the cellular lysis, precipitation, and removal of proteins and polysaccharides. The study reveals a dependence of the QRT-PCR measurement results on the DNA extraction method applied in the case of the construct-specific real-time PCR

detection method. Consequently, the robustness of each QRT-PCR method toward DNA extraction has always to be carefully investigated. The current practice to validate a complete measurement procedure for DNA in food or feed samples by incorporating a single DNA extraction method into the validation does only allow the assessment of this specific combination of extraction and detection method without further generalization.

Moreover and according to [33], six method combinations were collaboratively trialed to investigate the influence of the DNA extraction method and the real-time PCR detection method on the measured relative copy number of transgenic per endogenous sequences (Table 15.1). In the current study three different DNA extraction methods, namely, the cetyl trimethyl ammonium bromide (CTAB) method [59], the Wizard genomic DNA purification kit (Promega Benelux, Leiden, The Netherlands), and the GENE*Spin* kit (GeneScan Analytics GmbH, Freiburg, Germany), were compared with respect to their possible impact on the determination of the copy number ratio. Two real-time PCR detection methods, either construct- or event-specific, were evaluated. Whereas initially mostly construct-specific detection methods were developed [60], the increasing number of GM events authorized in Europe led to the decision to favor event-specific real-time PCR detection methods because of their ability to unambiguously identify the specific GM event. Both PCR methods used in this study passed successfully collaborative method validations according to ISO 21570. It has to be noted that matrix CRMs were used in these validations as unknown samples as well as for calibration and that only one DNA extraction method was applied during the validation of each method. However, ISO 21570 claims that also other DNA extraction methods can be used provided they can produce the same results. The results obtained in a specifically designed interlaboratory study revealed a significant influence of the DNA extraction method on measurement results when the MON 810 construct-specific QRT-PCR detection methods were applied. Statistical analyses confirmed the importance of validating DNA extraction methods in conjunction with real-time PCR methods.

In the other hand, Di Bernardo et al. [16] evaluated different DNA extraction procedures from food complex samples using PCR based methods targeting the reference genes *adh* and *lectin*. This study showed among the analyzed methods of extraction, the Qiagen kit gave a good-quality DNA only from simple foodstuffs, whereas the CTAB/PTB and Epicentre methods yielded the highest levels of DNA with a low level of quality. On the other hand, the Roche Kit was the most effective in recovering DNA from complex matrices. Another crucial study conducted by Cankar et al. [53], worked on the critical points of GMO quantification by real-time PCR and the effects of DNA extraction method and sample matrix on quantification. As it is known, the parameter or the indicator determining the reliability of quantitative results is the PCR efficiency in order to evaluate the quality and performance using different matrixes and extraction methods. The results showed variability of PCR efficiency within matrixes and these can lead to even large errors in quantification. The authors suggested that appropriate controls must be included in PCR quantification to evaluate the suitability of the isolated DNA for quantitative

GMO analysis. Finally, all the studies conducted showed that results were consistent each other and have indicated that both the quality and quantity of DNA recovered from food products tend to decrease with the extent to which the product is processed and the most suitable DNA extraction method strongly depends on the food matrix and that there is no "universal" method that could be used for all food samples and sometimes even different material from the same sample needs additional steps (potato tuber and leaf with or without CTAB precipitation step).

5 Conclusion

One of the challenges that the GMO analyst is facing and will face more and more in the future is the rapid pace of development of GM plants for a same taxon, the increasing number of taxa concerned by GM modifications as well as new and multiple genes and regulatory elements. In GMO analysis, the extraction methods used for the isolation of genomic DNA have a great influence in both quality and quantity of the recovered DNA. The selection and application of a specific DNA extraction method, in a particular laboratory, must take in account the requirements of the experimental work with respect to the samples type and as well as the cost and time. Validated and optimized protocols for the preparation of the nucleic acid samples for the detection and the quantification of GMOs needs to be more and more developed with interlaboratory trials in the all the international networks of GMO laboratories. These methods will be of a great utility for routine analysis.

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