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REVIEW

Effect of food processing on plant DNA degradation and PCR-based GMO analysis: a review

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Abstract The applicability of a DNA-based method for GMO detection and quantification depends on the quality and quantity of the DNA. Important food-processing conditions, for example temperature and pH, may lead to degradation of the DNA, rendering PCR analysis impossible or GMO quantification unreliable. This review discusses the effect of several food processes on DNA degradation and subsequent GMO detection and quantification. The data show that, although many of these processes do indeed lead to the fragmentation of DNA, amplification of the DNA may still be possible. Length and composition of the amplicon may, however, affect the result, as also may the method of extraction used. Also, many techniques are used to describe the behaviour of DNA in food processing, which occasionally makes it difficult to compare research results. Further research should be aimed at defining ingredients in terms of their DNA quality and PCR amplification ability, and elaboration of matrix-specific certified reference materials.

Keywords Food processing · DNA degradation · GMO detection · DNA quantification · PCR analysis

Introduction

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 [1, 2]. 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 [2–5], which may alter the result of a qualitative [6] and quantitative [7] GMO analysis.

Furthermore, 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. So-called matrix effects, plant polysaccharides and polyphenolics, feed additives, or reagents used in extraction procedures can be co-purified, which inhibits the PCR [1, 4, 7–10]. Sources of potential inhibitors and possible prevention actions are reviewed by Terry et al. [11].

DNA quality, purity and quantity for PCR

The suitability of isolated DNA as an analyte for PCR-based detection of GMOs will depend on the quality, purity, and quantity of the DNA.

It is well known that the efficiency of the PCR depends on DNA quality and purity. DNA quality is determined by its fragment length (or average molecular weight) and its degree of damage, and therefore varies according to the material under examination, the degree of processing, and the DNA extraction method applied [1, 12, 13]. DNA quality is normally assessed by a PCR test for an

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endogenous DNA sequence which is present in the plant genome, whether GM or not. This assay verifies the PCR quality status of the extracted DNA and guards against false negatives [14].

The purity of DNA can be severely affected by various contaminants in food matrices. The choice and optimisation of the DNA extraction procedures which eliminate potential inhibitory components and interfering substances may thus be of crucial importance for the success of a given DNA-based detection method [12, 15]. On the one hand, contaminants may originate from the material under examination, e.g. proteins, polysaccharides, lipids, and polyphenols [1, 16, 17]. On the other hand, chemicals used during the DNA extraction procedure such as cetyltrimethylammonium bromide (CTAB), EDTA, phenol, chloroform, SDS, ethanol, and isopropanol can cause inhibition of the PCR by inhibition of the *Taq* polymerase [13, 18–20]. A long list of salts, carbohydrates and other compounds frequently used in buffer solutions also reduce the performance of PCR [9, 18, 21]. For many situations, dilution of inhibited samples is a rapid and straightforward way of enabling amplification. This dilution exploits the sensitivity of PCR by reducing the concentration of inhibitors [9].

The purity of extracted DNA can be assessed by measurement of A_{260}/A_{280} and A_{260}/A_{230} UV absorption ratios with a spectrophotometer. When the 260/280 nm absorption ratio is between 1.5 and 2.0 and the 260/230 nm absorption ratio is more than 1.7 the extracted DNA should be suitable for PCR analysis [22, 23].

If the extracted DNA is of sufficient quality for PCR analysis, the only effect from the matrix would in theory be linked to the yield of the DNA extraction method. This is of importance, because the quantity of the DNA obtained will determine the DNA content that can be included in a PCR, which has an impact on the practical detection and quantification limit [24]. However, this is only true as long as the sample is homogeneous and extraction of DNA from all the particles is of similar efficiency. According to different studies, the quantity of template DNA used in PCR can range from 20 pg to 200 ng [16, 25–28]. If the amount of gene copies is insufficient for PCR amplification, the amount of template DNA used in the PCR may be increased [29, 30]. Too much DNA at the start of a PCR analysis should, however, be avoided, because this may result in a reduced PCR efficiency [31]. Moreover, the larger the genome size, the smaller the number of copies present in a fixed amount of DNA and thus the smaller the number of GM molecules present.

As a result of all these variables, evaluation of the effect of (GM) food processes on DNA degradation may depend on the extraction method used and the method chosen to evaluate DNA fragmentation. This review therefore not only focuses on food processes and their effect on DNA degradation and subsequent PCR-based GMO analysis, but also on factors affecting those DNA analyses (such as DNA quality, DNA quantity, target sequences and the complexity of food products) and on the methods used to evaluate DNA quantity and DNA degradation in GM food products.

DNA extraction

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. However, for a heterogeneous composite food product, particularly when several of the components may contain GM derivatives, e.g. pizza, the issue of sufficient homogenisation to ensure a representative sample is particularly critical, especially if a quantitative GM analysis is required [11]. Once a homogeneous and representative sample for a particular batch has been obtained, the analyst can choose among a vast range of methods. Many methods in GMO analysis are based on precipitation of the DNA using CTAB extraction buffer (cetyltrimethylammonium bromide) (Table 1). These methods are considered efficient for a wide range of plant-derived foods, in particular for separation of polysaccharides from DNA [13, 33, 34, 48]. Costs are lower than those for commercial kits because of the use of common chemicals (not taking the labour costs into account) [20, 34]. Other methods used are based on DNA binding to resins (Table 2) and magnetic particles (Table 3), prepacked glass fibres [43], non-chaotropic solid-phase extraction [48], use of PVPP [20, 35], and the FTA card, which can be immediately used as a PCR template [50]. So far it has been very difficult, or impossible, to obtain good quality DNA for PCR from highly processed food such as corn flakes, corn puffs, hydrolysed plant proteins (soya sauce), purified lecithin and starch derivatives (maltodextrins, glucose syrup) because of both PCR inhibitors and very low yields [1, 5, 54, 57, 58].

As a result of DNA degradation and the presence of PCR inhibitors, extraction of the DNA from processed foods is often a compromise between high yields and high purity [3]. 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 particular method should be chosen on a case-by-case basis. Tables 1, 2 and 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.

Table 1 DNA precipitation methods frequently used in GMO analysis

Method	Description	Samples	Other methods[a]	Ref.
AbiPrism 6100 PrepStation	Manufacturer's manual [32]	45 samples, including soya milk, wafer, soya meat, ice cream, soya cream, cracker, soya lecithin, biscuits, soya drink, soya oil, rice and soya wafers, corn flakes	R	[32]
CTAB extraction	[33]	Biscuits, chocolate	A, C	[34, 35]
	[36]	Soybean flour, soymilk, infant formula, beverage	A, U, C, N	[37]
	[25]	Soybean flour, soymilk, infant formula, beverage	A, U, C, N	[37]
	[25]	Miso	U	[38]
	[25]	45 samples	R	[32]
	[25]	Corn flour, corn starch	A, F, R	[28]
	[25]	Corn flour, canned maize, corn puff snacks, corn chip snacks, corn flakes, infant formula	A, U, N, R	[39]
	[25]	Soybean, soybean flour, soybean drinks, protein isolates, soybean sauce, tofu, soybean dessert, vegetarian soy products	U, F, C, R	[40]
	[41]	Miso	U	[38]
	[42]	Soybean flour, polenta, soymilk, soy bread, maize bread, fresella, cracker, chocolate snack	U, C	[43]
	[44]	Maize kernels	U, C, R	[45]
	[46]	Miso	U	[38]
	[47]	Flour, biscuits, instant paps	U, F, C	[48]
	[49]	Natto, soy sauce	U, C	[50]
Method D, E	Not specified	Maize kernels	U, C, R	[45]
MasterPure DNA purification kit	Manufacturer's manual [43]	Soybean flour, polenta, soymilk, soy bread, maize bread, fresella, cracker, chocolate snack	U, C	[43]

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

The type of extraction can significantly affect the measurement results of a PCR assay [7, 28, 51, 59–62]. Moreover, fractions of different particle size distribution may lead to unequal extraction efficiencies, which may lead to bias in GMO analysis results [44, 63–66].

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 [28, 34, 35, 67–71], which might change the overall extraction efficiency.

Other factors affecting the extraction efficiency are the presence of chemicals in the sample, for example fungicides [4], physicochemical changes during processing, which lead to binding of DNA to insoluble matrix components [7, 72, 73], oxidation or enzyme hydrolysis of DNA [74], and the length of the DNA to be extracted [28]. Food processes, for example thermal treatment, which lead to a decrease of DNA fragment length, will also result in changed DNA extraction efficiencies [75].

Target sequences

PCR is based on the amplification of one specific fragment which reflects the presence of a specific gene of interest. However, for amplification of one gene, many possibilities arise, resulting in different amplicons of different sizes. In Tables 4, 5 and 6, target sequences and their respective amplicon lengths for conventional and real-time PCRs, which are of importance to this review, are summarized. In the quantification of GMOs with real-time PCR two measurements are involved, because the GMO content reflects the proportion of the GM event-specific gene sequence to a reference gene. Here again, targeted gene sequences may vary in length and base composition.

Table 2 Resin-binding DNA extraction methods frequently used in GMO analysis

Method	Samples	Other methods[a]	Ref.
Chelex 100	Tofu, soybean flour, lecithin	A, U, C, N	[20]
DNeasy method	Tofu, soybean flour, lecithin	A, U, C, N	[20]
DNeasy plant mini kit	Polenta, crackers, tacos, tofu	A, U, C, R	[51]
	Corn and soya flour, biscuits, chocolate based products, miso, taco shells, soya protein	U, C	[52]
	Corn flour, corn starch	A, F, R	[28]
DNeasy tissue kit	Flour, feed, oil	C	[53]
DNeasy plant maxi kit	Corn starch	A, F, R	[28]
GenElute plant genomic kit	Corn flour, canned maize, corn puff snacks, corn chip snacks, corn flakes, infant formula	A, U, N, R	[39]
GeneSpin DNA isolation kit	Biscuits, chocolate	A, C	[34]
	Soybean, soybean flour, soybean drinks, protein isolates, soybean sauce, tofu, soybean dessert, vegetarian soy products	U, F, C, R	[40]
	Flours, biscuits, instant paps	U, F, C	[48]
Method A, B	Maize kernels	U, C, R	[45]
NucleoSpin food kit	Miso	U	[38]
	Polenta, crackers, tacos, tofu	A, U, C, R	[51]
	Corn flour, corn starch	A, F, R	[28]
	Soybean, soybean flour, soybean drinks, protein isolates, soybean sauce, tofu, soybean dessert, vegetarian soy products	U, F, C, R	[40]
	Miso	U	[38]
	Soy flour, polenta, soymilk, soy bread, maize bread, fresella, cracker, chocolate snack	U, C	[43]
Nucleon PhytoPure kit	Biscuits, chocolate	A, C	[34]
	Tofu, soybean flour, lecithin	A, U, C, N	[20]
Plant genomic DNA extraction miniprep system	Miso	A, F, R	[38]
QIAamp DNA stool mini kit	Biscuits, chocolate	A, C	[34]
	Corn flour, canned maize, corn puff snacks, corn chip snacks, corn flakes, infant formula	A, U, N, R	[39]
	Corn and soya flour, biscuits, chocolate based products, miso, taco shells, soya protein	U, C	[52]
	Soybean flour, polenta, soymilk, soy bread, maize bread, fresella, cracker, chocolate snack	U, C	[43]
	Polenta, crackers, tacos, tofu	A, U, C, R	[51]
Wizard method	Maize flour, polenta	A, U, N, R	[39]
	Tofu, soybean flour, lecithin	A, U, N	[20]
	Polenta, crackers, tacos, tofu	A, U, C, R	[51]
	Canned maize, corn snacks, corn flakes, infant formula	A, U, N, R	[39]
	Various foodstuffs	U, C	[54]
	Soybean flour, soybean proteins	C, N	[55]
	Corn flour, canned maize, corn puff snacks, corn chip snacks, corn flakes, infant formula	A, U, N, R	[39]
	Various foodstuffs	C, R	[56]

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

For analysis of processed food products, short fragments may be targeted, because they are more stable than long fragments [29, 80, 123]. DNA regions containing high GC contents are generally also regarded to be stable when exposed to high temperatures [29, 83]. This has clearly been shown by Chen et al. [80], who have shown that the transgenic content during soymilk production decreased from 2.5% to 1.6%, because the targeted

Table 3 DNA extraction methods based on magnetic particles used in GMO analysis

Method	Samples	Other methods[a]	Ref.
Method C	Maize kernels	U, C, R	[45]
Wizard magnetic + Kingfisher	Corn flour, corn starch	A, F, R	[28]
Wizard magnetic DNA purification for food	Corn flour, corn starch	A, F, R	[28]
	Corn flour, canned maize, corn puff snacks, corn chip snacks, corn flakes, infant formula	A, U, N, R	[39]
	Soybean, soybean flour, soybean drinks, protein isolates, soybean sauce, tofu, soybean dessert, vegetarian soy products	U, F, C, R	[40]
	Feed, maize flour, maize oil	C	[53]
	Biscuits, chocolate	A, C	[34]

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

endogenous gene is relatively more stable than the exogenous gene. Thus, the GC content of the target sequence of recombinant DNA may be an indicator of stability or fragmentation of the target gene in processed foods [124].

Particularly in GM quantification analysis, both detections should have the same PCR efficiency as the standard curve, because high fluctuation in PCR efficiency could lead to over or underestimation of GMO content [7, 64]. Finally, in samples with small amounts of gene copies, measurement uncertainty of GMO estimation by real-time PCR increases because of the high variability of quantity estimates caused by the occurrence of stochastic effects [7].

Complexity of food products

Composed products consist of several ingredients with different properties. The nature of each ingredient will determine its extraction efficiency (e.g. oil versus flour) which may alter during processing. As laboratories rarely receive complete compositional data on the products, the choice of a specific extraction technique is usually based on previous experience with similar samples [7]. Furthermore, food products often contain different components derived from the same species, which will be regarded as a single ingredient in the framework of the European 1830/2009/EC GMO labelling rules [125]. With each tissue having a different DNA contribution, and different levels of DNA degradation and/or a depletion mixed in variable unknown ratios, precise quantitative analysis is impossible [3, 126–128].

Another limitation of DNA-based GMO detection and quantification in food products is related to the complex zygosity of certain crops, especially corn. With the endosperm being triploid, the embryo diploid, and the pericarp haploid, the DNA content of each type of tissue differs [129]. The endosperm fraction is milled and is primarily used as raw material for processed foods, for example corn snacks, after removal of the embryos. To produce corn starch, the seed coat, embryo, and protein-rich corn meal fraction are removed from the corn grains [29]. As a result, the measured GMO content of starch will be different from the measured GMO content of the kernel as a whole.

Susceptibility of DNA to processing

Temperature

The affect of high temperatures on DNA degradation is most obvious and has, therefore, attracted most attention by researchers. Temperature processing results in the physical degradation of DNA (denaturation). The mechanism of DNA destruction by heat is based on depurination or deamination. Although at temperatures above 100 °C significant strand scission and irreversible loss of secondary structure occurs [74, 130], normal temperatures used for processes such as canning or even autoclaving at 121 °C for 15 min do not destroy all the DNA available for PCR [25]. Next to cooking, baking, drying and roasting are heat processes which degrade DNA. The DNA becomes sheared into smaller fragments and, therefore, PCR sensitivity will be reduced.

Drying

Even at 70 °C DNA is degraded, as shown during the drying of potato sticks for 2 h [73]. At higher temperatures, degradation of DNA is observed after much shorter processing times. When corn grains are heated at 94 °C for 5 min, DNA is degraded, resulting in the complete absence of the 577 bp amplicon [100]. The drying of wet-

Table 4 Conventional PCR methods used to evaluate the effect of food processing on soy DNA degradation

Process	Details	Target and length	Cycles	DNA in PCR	Other methods	Ref.
Cooking of seeds	80–100 °C, 15–360 min	*Lec*: 913 bp [66]	40	2 μL	A, U	[66]
Cooking of DNA solutions	100 °C, 10–180 min	*Lec*: 318, 444, 682 bp [76] RRS: 509, 180 bp [76]	n.d.	Various amounts	N	[76]
Cooking of fermented tofu	10 min, in a rice cooker for 10 min, in hot oil for 2 min	*Lec*: 318, 444, 682 bp [76] RRS: 509, 180 bp [76]	n.d.	Various amounts	N	[76]
Soymilk production	Soaking, grinding, filtering, boiling for 10 min	*Lec*: 118 [77], 714 [69], 1060 bp [78] RRS: 172 [55], 564 [78], 1339, 1719 bp [69]	35	1 μL	–	[2]
Soymilk production	Soaking, rinsing, grinding, filtering, boiling for 10 min	*Lec*: 118 [77], 714 bp [69], 1060 bp [78] RRS: 172 [55], 564 [78], 1339 [69], 1482 [69], 1719 bp [69]	38 35	1 μL	–	[69]
Soymilk production	Soaking, jordaning, boiling at 100 °C for 15 min, blending, homogenisation, sterilization at 121 °C for 30 s	*Lec*: 162, 407, 836, 1883 bp [79] RRS: 190, 408, 807, 1512 bp [79]	35 40	100–200 ng	R	[80]
Bean curd production	Blending, filtration, cooking at 100 °C for 15 min with 15% $CaSO_4$, squeeze-moulding	*Lec*: 162, 407, 836, 1883 bp [79] RRS: 190, 408, 807, 1512 bp [79]	35 40	100–200 ng	–	[79]
Microwaving of flour	540–900 W, 2 min	*Lec*: 118, 413 bp [81] 35S: 172 bp [82], 181 bp [83] *Nos*: 121 bp [83]) RRS: 101 bp, 210 [83]	40 40 40 40	80–1000 ng	A, U	[83]
Sterilisation of soymilk	121 °C, 30 s	*Lec*: 162, 407, 836, 1883 bp [79] RRS: 190, 408, 807, 1512 bp [79]	35 40	100–200 ng	–	[79]
Autoclaving of seeds	121 °C, 15 min	*Lec*: 95, 195, 295, 395, 495, 595 bp [84] RRS: 101, 201, 402, 513 bp [84]	40 40	50 ng	–	[84]
Autoclaving of flour	121 °C, 20 min	*Lec*: 100 bp [50], 118 bp [78, 85] RRS: 100, 110, 120, 130, 140, 150, 180 bp [78, 85, 86]	40 40	2 μL	A, U	[86]
Autoclaving of DNA solutions	121 °C, 10–30 min	*Lec*: 318, 444, 682 bp [76] RRS: 180, 509 bp [76]	n.d.	Various amounts	N	[76]
Autoclaving of flour	121 °C, 15 min	*Lec*: 118, 413 bp [81] 35S: 172 bp [82], 181 bp [83] *Nos*: 121 bp [83] RRS: 101 bp, 210 [83]	40 40 40 40	80–1000 ng	A, U	[83]
Spray drying of soybean filtrate from bean curd production	160 °C	*Lec*: 162, 407, 836, 1883 bp [79] RRS: 190, 408, 807, 1512 bp [79]	35 40	100–200 ng	–	[79]
Baking of cookies with soybean flour	205 °C, 10 min	*Lec*: 118 bp [55] 35S: 123 bp [4] *Nos*: 118 bp [4] RRS: 177 bp [87]	35 50 50 35	2 μL 1 μL 1 μL 2 μL	A, U, R	[6]
Baking of flour	220 °C, 30 min	*Lec*: 913 bp [66]	40	2 μL	A, U	[66]

Table 4 (continued)

Process	Details	Target and length	Cycles	DNA in PCR	Other methods	Ref.
Baking of bread with soybean flour	Rising and baking at 220–240 °C for 40 min	*Lec*: 118 bp [88] RRS: 172 bp [77]	35 35	10–100 ng	A	[89]
Baking of bread with soybean flour	Rising and baking at 200–230 °C for 20 min	*Lec*: 118 bp [55] 35S: 123 bp [4] *Nos*: 118 bp [4] RRS: 177 bp [87]	35 50 50 35	2 μL 1 μL 1 μL 2 μL	A, U	[90]
Roasting and deep roasting of beans		*Lec*: 95, 195, 295, 395, 495, 595 bp [84] RRS: 101, 201, 402, 513 bp [84]	40 40	50 ng	–	[84]
Frying of tofu	175 °C	*Lec*: 95, 195, 295, 395, 495, 595 bp [84] RRS: 101, 201, 402, 513 bp [84]	40 40	50 ng	–	[84]
Decreased pH of flour	pH 4.75 at 65 °C	*Lec*: 118 [77], 714 bp [69], 1060 bp [78] RRS: 172 [55], 564 [78], 1339 [69], 1482 [69], 1719 bp [69]	38 35	1 μL	–	[69]
Miso production	Fermentation up to 180 days	*Lec*: 118 bp [91] 35S: 195 bp [25] *Nos*: 180 bp [25] RRS: 172 bp [91]	40 40 40 40	5 ng	A, N	[38]
Miso production	Fermentation up to 210 days	RRS: 509, 180 bp [76]	n.d.	Various amounts	N	[76]
Sufu production	Fermentation up to 180 days	RRS: 180, 509 bp [92]	30 and 30	1 μL	N	[93]
Natto production	Fermentation	*Lec*: 100 bp [50], 118 bp [78, 85] RRS: 100, 110, 120, 130, 140, 150, 180 bp [78, 85, 86]	40 40	2 μL	A, U	[86]
Tofu production	Acid and salt addition of soymilk, filtration	*Lec*: 118 [77], 714 [69], 1060 bp [78] RRS: 172 [55], 564 [78], 1339, 1719 bp [69]	35 40	1 μL	–	[2]
Tofu production	Soaking, grinding, soaking, filtering, salt addition, boiling, squeeze-moulding	*Lec*: 95, 195, 295, 395, 495, 595 bp [84] RRS: 101, 201, 402, 513 bp [84]	40 40	50 ng	–	[84]
Refining of soybean oil	Degumming, neutralization, bleaching, deodorization	*Lec*: 118 bp [55]	35	2 μL	–	[94]
Refining of soybean oil	Degumming	*Lec*: 118 bp [55]	35	2 μL	A, R	[68]
Refining of soybean oil	Centrifugation of commercial oil, spiked with a DNA-solution	*Lec*: 414, 118 bp [55]	25 and 40	2 μL and 2 μL	N	[95]
Irradiation of beans	500, 800 and 1000 Gy	RRS: 180 bp [92]	35	2 μL	–	[96]

35S: cauliflower mosaic virus 35S promoter, A: agarose gel electrophoresis, *Lec*: lectin, N: nested PCR, *Nos*: nopaline synthase, R: real-time PCR, RRS: Roundup Ready soy (targeting an inserted gene or junction fragment), U: UV spectrophotometry, n.d.: not determined/mentioned

milled gluten fractions and hydrated kernels, by use of increased temperatures up to 135 °C for 2 h, severely degrades DNA [58]. The application of temperatures above 100 °C during the production of flaked corn (including rolling) causes substantial degradation of the DNA, as shown by agarose gel electrophoresis [100]. So heating processes leads to the degradation of intact DNA. However, this does not necessarily render PCR amplification impossible. In an experiment, in which canola meal was pelleted at 100 °C, detection of a 1363-bp fragment remained possible [131].

Table 5 Conventional PCR methods used to evaluate the effect of food processing on maize DNA degradation

Process	Details	Target and length	Cycles	DNA in PCR	Other methods	Ref.
Freezing of flour	−80 °C, 48 h	*Zein*: 84 bp [97] MON810: 149, 401 bp [98], 170 bp [82], 194 bp [99], 95 bp [83]	40 40	80–1000 ng	U	[83]
Drying of grain	90 °C, 5, 10, 15 min 92 °C, 5 min 94 °C, 5 min	*RbcS*: 577 bp [100]	35	1 μL	A	[100]
Cooking of grain	100 °C, 3–120 min	*Zein*: 329 bp [101]	40	100 ng	–	[102]
Microwaving of flour	540 W and 900 W, 2 min	*Zein*: 84 bp [97] MON810: 149, 401 bp [98], 170 bp [82], 194 bp [99], 95 bp [83]	40 40	80–1000 ng	U	[83]
Steeping, wet-milling and processing	Steeping, wet-milling, and heating at 135 °C for 2 h	*RbcS*: 1660 [103], 1000 [104], 900 [105], 445 bp [106]	60	5 μL	A	[58]
Corn masa production	Alkaline boiling with 1% lime for 20 min, drying, milling	*Cry1A(b)*: 585, 957, 1416 [69], 1914 bp [107]	38	1 μL	–	[2]
Polenta production	Grinding, boiling in 0.4% NaCl for 105 min	CryIA(b): 1914 bp [107], 211 bp [108]	38	1 μL	–	[108]
Autoclaving of grain	120 °C, 10–60 min	*Zein*: 329 bp [101]	40	100 ng	–	[102]
Autoclaving of flour	121 °C, 15 min	*Zein*: 84 bp [98] MON810: 149, 401 bp [98], 170 bp [82], 94 bp [99], 95 bp [83]	40 40	80–1000 ng	U	[83]
Baking of flour	180 °C, 15 min	*Zein*: 84 bp [97] MON810: 149, 401 bp [98], 170 bp [82], 194 bp [99], 95 bp [83]	40 40	80–1000 ng	U	[83]
Baking of bread with maize flour	200–250 °C, 55 min 180 °C, 30 min	*Ivr*: 226 bp [109] CryIA(b): 211 bp [108]	42 42	n.d.	A	[110]
pH changes of flour	pH 2 and pH 9	CryIA(b): 1914 bp [107], 211 bp [108]	38	1 μL	–	[108]
Heating and pH changes of flour	85 °C/pH 8.4 and 65 °C/pH 4.0	*Cry1A(b)*: 585, 957, 1255, 1416 [69], 1914 bp [107]	38	1 μL	–	[69]
Ensiling of grain	Chopping, stored at pH 3.9–4.1 for 216 days	*Ivr*: 226 bp [109] *Cry1A(b)*: 1914 bp [107], 211 bp [108]	38 38	100 ng or 1 μL	A	[111]
Ensiling of grain	Chopping, stored at pH 3.8–5.5 for 61 days	*RbcS*: 173, 896, 1197, 1753 bp [112] *Cry1A(b)*: 211 bp [108], 420 bp 98], 727, 1423 bp [112]	35–40	150 ng	–	[112]
Ensilage of grain	n.d.	*RbcS*: 577 bp [97]	35	1 μL	–	[65]

A: agarose gel electrophoresis, AmpR: ampicilin resistance, *Bt*: *Bacillus thuringiensis*, *cry1A(b)*: Bt-toxin, *RbcS*: chloroplast enzyme rubisco, MON810: fragment of the *hsp70* intron1-*cryIA*(b) insert (gene or junction fragment), *ivr*: invertase, U: UV spectrophotometry, n.d.: not determined/mentioned

Cooking

The effect of water during heat treatment has been examined recently by Murray et al. [67] by comparing the effect of temperature treatment of 100 °C for up to 60 min on dry and wet soybean meal samples. For the dry samples, there were no clear changes in estimated available template DNA content, whereas reduction in the amount of DNA detectable using the longest assays (830 bp and 1022 bp) was evident for the wet soy meal samples.

Heating of DNA solutions is the easiest way of examining its effect on DNA fragmentation, by varying processing time and temperature. Hupfer et al. [132] have shown that after 60 min of heat treatment at 95 °C the average DNA fragment length in a DNA solution is reduced to less than 600 bp. Similarly, Debode et al. [116] observed that the mean size of the DNA segments is still about 400 bp after the heating of DNA solutions at 99 °C for 7 h in a heating block. Microwave treatment of DNA solutions for 0–15 min at maximum power of 800 W, with maximum heating periods of 3 min each time and intermediate cooling, yielded more severely degraded material DNA, but the amount of DNA segments with size larger than the target was still great [116]. Nevertheless, even temperatures lower than the cooking temperature may result in degradation of plant DNA. During the cooking of potato tubers for 1 h at 80 °C, DNA was degraded to fragments smaller than 792 bp [2].

The effect of boiling temperature on DNA degradation has been studied with soymilk production as a typical food process. During the processing of soymilk, soybeans are soaked overnight, ground, and filtered. The resulting filtrate

Table 6 Real-time PCR methods used to evaluate the effect of food processing

Process	Details	Target and length	DNA in PCR	Other methods	Ref.
Drying of potato tubers and sticks	70 °C, 2 h	*Gbss*: 96, 325, 719 bp [73]	5 μL	–	[73]
Heating of potato tubers	80 °C, 1 h	*Gbss*: 96, 325, 719 bp [73]	5 μL	–	[73]
Heating of maize flour	95 °C, 5 s–30 min	*SSIIb-3*: 114 bp [29] NK603: 108 bp [113]	50 ng	A, U, F	[114]
Cooking of soy DNA solutions	99 °C, 1–7 h	*Lec*: 80 bp [115] RRS: 170 bp [6]	5 μL	A	[116]
Cooking of maize meal	100 °C, 5–60 min	G3PD: 68, 170, 472, 967 bp [75] INCW2: 97, 331, 581, 980 bp [75]	5 μL	A	[75]
Dry cooking of soybean meal	100 °C, 60 min	*Lec*: 98, 189, 529, 830 bp [67] TDF5: 86, 193, 491, 1022 bp [67]	5 μL of diluted sample	A	[67]
Wet cooking of soybean meal	100 °C, 5–60 min	*Lec*: 98, 189, 529, 830 bp [67] TDF5: 86, 193, 491, 1022 bp [67]	5 μL of diluted sample	A	[67]
Polenta processing	Boiling of maize flour in 0.4% NaCl for 45 min	*Zein*: 72 bp [39]	10 μL	A	[39]
Tofu production	Soaking, grinding, heating to 94 °C for 7 min, straining, heating to 85 °C in $MgCl_2$ for 20 min, pressing	*Lec*: 98, 189, 529, 830 bp [67] TDF5: 86, 193, 491, 1022 bp [67]	5 μL of diluted sample	A	[67]
Soymilk production	Soaking, grinding, filtration, boiling at 100 °C for 30 min, cooling	*Lec*: 74 bp [117] RRS: 74 bp [117]	2 μL	–	[117]
Soymilk production	Soaking, jordaning, boiling at 100 °C for 15 min, blending, homogenisation, sterilization at 121 °C for 30 s	*Lec*: 118 bp [80] RRS: 85 bp [118]	100–200 ng	–	[80]
Nixtamal (corn masa) and masa flour production	Cooking at 87 °C for 45 min, steeping in 0.44% $Ca(OH)_2$ at 70–75 °C for 3.5 h, rinsing (= nixtamal), grinding, drying at 130 °C, grinding (= masa flour)	CBH351 Starlink corn (commercial kit, confidential)	100 ng	A, U	[72]
Microwave heating of potato	10, 25, 50 kGy	*Gbss*: 96, 325, 719 bp [73]	5 μL	–	[73]
Heating of soybean flour	110 °C, 5–60 min	*Lec*: 118 bp and 89 bp [29]	25 ng	A	[29]
Heating of maize flour	110 °C, 5–60 min	*SSIIb*: 151, 133, 114, 83 bp [29] MON810: 113 bp [119] 35S: 101 bp [119]	25 ng	A	[29]
Autoclaving of soy DNA solutions	Oven drying at 55 °C, autoclaving at 120 °C for 10–80 min	*Lec*: 80 bp [115] RRS: [6] 170 bp	5 μL	A	[116]
Autoclaving of soymilk	121 °C, 15–35 min	*Lec*: 74 bp [117] RRS: 74 bp [117]	2 μL	–	[117]
Heating of potato flakes	150 °C, 1 min	*Gbss*: 96, 325, 719 bp [73]	5 μL	–	[73]
Heating of potato chips	175 °C, 3 min	*Gbss*: 96, 325, 719 bp [73]	5 μL	–	[73]
Extrusion of maize meal	60 and 170 °C, torque settings 6–36 Nm	G3PD: 68, 170, 472 and 967 bp [75] INCW2: 97, 331, 581 and 980 bp [75]	5 μL	A	[75]
Tortilla chips production	Soaking, pressing, hot plate cooking at 200 °C and frying at 190 °C for 60 s	CBH351 Starlink corn (commercial kit, confidential)	100 ng	A, U	[72]
Corn chips production	Soaking, pressing and frying at 190 °C for 60 s	CBH351 Starlink corn (commercial kit, confidential)	100 ng	A, U	[72]
Dry-corn chips production	Soaking, pressing, oven drying at 70 °C and frying at 190 °C for 60 s	CBH351 Starlink corn (commercial kit, confidential)	100 ng	A, U	[72]
Baking of cookies with soybean flour	205 °C, 10 min	RRS (commercial kit, confidential)	1 μL	A, U, C	[6]

Table 6 (continued)

Process	Details	Target and length	DNA in PCR	Other methods	Ref.
Ensilage of grain	4 weeks	Plant: 199 bp [120], *ivr*: 226 bp [109], *zein*: 275 bp [101], *cry1A(b)*: 211 bp [108], 16S rDNA: 1490 bp [121], AmpR: 810 bp [122]	60–90 ng	SybrGreen	[122]
Refining of soybean oil	Degumming	RRS (commercial kit, confidential)	1 μL	A, C	[68]
Sonication of maize flour	20 kHz, 5 min	*SSIIb-3*: 114 bp [29] NK603: 108 bp [113]	50 ng	A, U, F	[114]
Sonication of soy DNA solutions	170 W, 5–480 min	*Lec*: 80 bp [115] RRS (35S-CTP): 170 bp [6]	5 μL	A	[116]

35S: cauliflower mosaic virus 35S promoter, A: agarose gel electrophoresis, AmpR: ampicilin resistance, C: conventional PCR, F: fluorescence measurement, G3PD: glyceraldehyde-3-phosphate dehydrogenase; *gbss*: granule-bound starch synthase, INCW2: cell wall invertase, *ivr*: invertase; *lec*: lectin, MON810: fragment of the *hsp70* intron1-*cryIA*(b) insert (gene or junction fragment), RRS: Roundup Ready soy (inserted genes or junction fragment), *SSIIb*: starch synthase, TDF5: leucine-zipper-like protein, U: UV spectrophotometry, n.d.: not determined/mentioned

is then boiled to obtain soymilk [2]. According to Kharazmi et al. [2], DNA degradation is already severe after mechanical treatment of soaked soybeans before heating. Remarkably, the effect of the syruping, the heating of the soaked beans at 100 °C for 10–20 min, is rather small [2, 80]. Similarly, research by Chen et al. [79] on the effect of cooking at 100 °C for 15 min during bean curd formation did not show any remarkable effects on the DNA. And after boiling during the production of tofu, okara, and soymilk, Ogasawara et al. [84] could still detect a 595-bp fragment.

Chemical treatment during the boiling process might add to the destruction of DNA. During corn masa production, cornmeal is mixed in 1% lime solution and boiled for 20 min at 100 °C, the combined effect contributes to degradation of DNA to fragments smaller than 585 bp [2]. Nevertheless, no further degradation could be observed by Kharazmi et al. [2] after acid and salt ($CaCl_2$) precipitation of proteins in soymilk for tofu production. Amplified fragments up to 595 bp could also be observed in tofu by Ogasawara et al. [84]. The additional effect of salts has also been studied during polenta production. After the boiling of maize flour in an aqueous solution of 0.4% (*w*/*v*) sodium chloride for 30 min, a 1914-bp fragment could no longer be detected, whereas a 211-bp fragment was still present even after 105 min of thermal treatment [108]. Gawienowski et al. [58] noticed that the addition of sulfur dioxide during steeping, without any heat treatment, degraded DNA, because of metabolic activity.

These results shows that a temperature of 100 °C does not seem to degrade DNA very severely. However, Murray et al. [75] observed that cooking of corn meal for 60 min resulted in a clear decrease of the DNA content. Next to the destructive effect of the heat on the DNA, Murray et al. [67, 75] suggested that this could also be because of reduced recovery of high-molecular-weight DNA by extraction. The time of the treatment will also be important, because the longer the heat treatment, the greater the fragmentation of the DNA [29, 76, 83].

Autoclaving

The autoclaving of food products at 121 °C has been shown to have a stronger degrading effect on DNA than cooking. After autoclaving of soybeans at 121 °C for 15 min, Ogasawara et al. [84] only obtained DNA fragments shorter than 295 bp. In real-time PCR detection, autoclaving might lead to an increase of the cycle threshold (Ct) value with 8.5 cycles after 60 min treatment at 120 °C [116]. The sterilisation process also involves higher pressure, which adds to the destructive effect of the DNA [80, 84]. Forbes et al. [65] showed that high-pressure steam (>100 °C, >1 bar) for times ranging from 30 s to 20 min resulted in severe or complete DNA degradation, whereas lower-pressure steam (85 °C, <0.5 bar) for 10 min resulted in partial degradation of the DNA. High pressures may also be combined with high temperature during extrusion. At 170 °C, extrusion has a clear effect on DNA degradation, because PCR amplification of a 68-bp fragment was impossible by real-time PCR [75].

Baking

Different baking experiments at 200, 220, and 230 °C show that baking substantially reduces the size of the extracted DNA [6, 66, 89, 90, 128, 133]. Sampling may be important in the analysis of bread samples. The top crust is directly exposed to heat by convection, which results in a more intense heating compared with the bottom crust, which is heated through the tray by conduction. Also, the higher moisture content in the centre of the bread contributes to greater DNA degrada-

tion than for a sample just taken under the crust [66, 90]. Moreover, the extractability of the still available DNA might change through the duration of the baking process, because of changes in the microstructure of the bread, in particular in the early stages of the baking [90, 128].

Roasting

The effect of roasting on soybean samples was investigated by Ogasawara et al. [84]. In roasted soybean (iridaizu), detection of a 595-bp fragment was possible. But only a DNA fragment of 195 bp from pulverising roasted soybean (kinako) could be amplified. Because of the pulverisation, the effect of the roasting process is much more severe, penetrating the centre of the kinako samples [84].

Frying

In the course of potato chip and flake production, respectively, plant DNA was strongly degraded by heat treatment at 175 °C for 3 min and at 150 °C for 1 min, and only DNA fragments of 96 bp were amplified from the DNA isolated from the final products [69]. However, the effect of frying abura-age (sliced and fried tofu) at 175 °C seemed less severe, because amplification of a 595-bp PCR fragment was possible [84]. Furthermore, Quirasco et al. [72] compared the effect of deep-frying humid masa with oven dried masa. The DNA from the deep-fried masa was much less degraded. Apparently, the presence of moisture buffers the thermal shock of frying.

pH

Low pH

Many foods, for example fruits or vegetables, are characterised by acidic pH conditions or are subjected to lower pH, for example during the preparation of tomato juice [108]. Acidic pH depurinates DNA and leads to subsequent strand cleavage [109, 134]. Acid-catalysed reactions are accelerated by simultaneous heat treatment [108, 135], as shown for tomato serum at pH 4.3 and 65 °C, and for soybean flour solutions at pH 4.75 and 65 °C [69]. pH-dependent breakdown of DNA has also been observed during rising of bread dough [89, 110, 128], and wet-milling of corn, including soaking of the kernels in a weakly acidic solution, is likely to be more destructive to the DNA than milling alone [28].

The effect of pH may be limited, however, because of cell wall structures protecting the DNA from cleavage. Detection of DNA template after prolonged incubation at low pH suggests that after initial cell lysis and preliminary DNA destruction, the enzymes responsible for DNA degradation (endogenous nucleases) are destroyed more quickly than DNA itself and its further breakdown is avoided [130]. Moreover, DNA is denatured by extremes of pH into single-stranded DNA, which can remain a substrate for PCR. Under strongly acidic (pH 3) conditions, depurination of DNA can occur, leading to nicks in the DNA strands and unsuccessful PCR.

The natural decrease in pH during ensiling of maize has been studied by Hupfer et al. [111]. It was shown in feed production that DNA was degraded during ensiling of *Bt*-176 corn (pH 3.9–4.1), and fragments of 1914 bp were no longer detectable after 106 days. Aulrich et al. [136] were able to detect a 194-bp fragment in whole plant silage and maize cob mixed silage over the entire duration of 200 days of ensiling, whereas Einspanier et al. [122] reported that the concentration of plant genes decreased to a level of 1.3–3% of the original concentration after ensiling of corn. Similar results were obtained by Lutz et al. [112]. However, the degradation of DNA during ensiling should not only be attributed to a reduction of the pH alone, but also to the action of endogenous nucleases of the plant and/or exogenous nucleases of the microflora [2, 111, 112].

Failures in extracting detectable levels of DNA have been reported for distilled ethanol, a process including mechanical stress, enzymatic hydrolyses, microbial fermentation, and thermal treatment. In particular the distillation process at reduced pH degrades the DNA significantly [123, 137].

High pH

In contrast, DNA is relatively stable at alkaline pH. At pH 8.5–9.5, the double strands, of the DNA molecule are separated, but only is broken down [108]. Strongly alkaline solutions may be present in the initial stages of the preparation of tortilla and hominy from maize. Specific attention has therefore been paid to the degradation of DNA as a result of the combined effect of pH and temperature, such as during the alkaline boiling (pH 11.0) of corn meal. Alkaline-cooked corn, called nixtamal or corn masa, is an instant product for the production of Mexican corn-based foods for example tortillas, corn chips, taco shells, and tamales [72]. Kharazmi et al. [2] reported the failure of amplification of fragments greater than 585 bp, whereas Hupfer et al. [108] still detected 1914-bp DNA fragments of corn after boiling at pH 9.0 for 60 min. In a real-time quantitative PCR approach, Quirasco et al. [72] were able to detect and quantify up to 0.1% StarLink corn after alkaline cooking, despite progressive degradation of genomic DNA during processing.

A combined effect of low and high pH is obtained in processing of soybean protein isolates and concentrates. After alkaline lysis of degreased soybean grits, soluble components

are removed and proteins are precipitated at low pH. In most cases, the protein isolate is then spray-dried [69]. DNA fragments of various lengths, in some cases even up to 1000 bp, which result in positive amplification, can be observed in protein isolates [15, 69, 90, 138].

Fermentation

The results of Pan and Shih [38] revealed that detection of transgenic components of a type of miso decreased gradually until the 120th day of fermentation, and from that point the 35S promoter could not be detected. When fermentation of miso is nearly complete, i.e. after 5–6 months, most of the DNA fragments are degraded to 200 bp or below [38, 76]. Nevertheless, a 95-bp amplicon could be successfully detected in miso by use of conventional PCR [84] and by use of a nested PCR [76]. The strong DNA degradation during the fermentation process might be explained by the digestion of DNA through DNases derived from microorganisms [84]. Similar difficulties in obtaining positive PCR results were observed in the production of sufu, a fermented tofu product. Nested PCR had to be used to enable positive amplification [83]. Furthermore, in the fermented soy products natto (fermented soybeans) and soy sauce genomic DNA is degraded during the fermentation process [84, 86].

Mechanical treatments

With the handling of raw materials, shear forces may be one of the first forces initiating DNA fragmentation. Forbes et al. [65] and Chiter et al. [100] analysed the effect of grinding and milling on wheat samples and could not observe any significant effect on the average molecular weight of the DNA isolated. Chen et al. [79, 80], however, reported degradation of DNA as a result of grinding, blending, homogenisation, and squeeze-moulding of Roundup Ready soybean samples, although grinding seemed to have the greatest effect [79]. According to Kharazmi et al. [2], the mechanical step of grinding of soaked soybeans is a more crucial DNA-degrading step than the heat treatment during the production of soy milk and tofu. Similarly, Murray et al. [67] observed increased DNA degradation of drained and blended soybeans overnight, because of metabolic activity in the seed. Tilley [128] showed that the mixing and punching stages during breadmaking have a discernable effect on DNA size, indicated by a slight decrease in molecular weight.

Shear-induced degradation may be caused not only by processing of the food but also in the many processing steps in extraction and purification of the DNA [139]. This shear stress may be associated with stirring, centrifugation, pumping, filtration, pipetting, spray drying, vial filling, and nebulisation [139]. There is, however, little specific research on the stability of DNA throughout shear-induced processes and on subsequent PCR detection. Chen et al. [79] reported that spray-drying, physical shearing, high temperature, and sudden high pressure cause distinct and rapid degradation of DNA. Furthermore, Herman et al. [140] observed substantial shearing of the DNA during chocolate production [140], which could have an effect on the detection of soy lecithin.

Enzymatic degradation

Klein et al. [141] investigated the elimination of nucleic acids during the manufacture of sugar. Intermediate and end products were analysed for the presence of DNA via PCR. Southern blot hybridisation of the targeted sequences delivered positive signals in samples from raw juice only, but not in those from carbonation sludges, thin and thick juices, or white sugar from transgenic beets. These results reveal already severe degradation of nucleic acids in the first steps of processing. The degradation of the DNA was ascribed to the enzymatic activity of sugar beet endonucleases, and to irreversible adsorption on the sludge, precipitation, hydrolysis because of the high temperatures in the carbonation and evaporation steps, and as a result of the exclusion of DNA in the crystallisation step.

Degradation of DNA was also observed by Hupfer et al. [111] during ensilage, actually because of the chopping of plant tissue, which results in disruption of cell walls and membranes. As a result, DNA and nucleases are released leading to the degradation of the DNA [2, 111]. Moreover, the pH decreases as a result of lactic acid fermentation, which accelerates the degradation of DNA [111].

Nuclease-induced DNA degradation may also explain why DNA fragments decrease over time during bread dough rising [90, 110]; it may also have some effect during the storage of fresh food [80]. Differences in the endogenous DNase content of a soybean or maize grain or in the compartmentalisation of DNase in grain should also be taken into account when analysing the effect of processing on DNA degradation [67].

Oil extraction and refining

The effect of oil refining on the detection of DNA has been studied on soybean, rapeseed and corn oil. In cold-pressed rapeseed oil, PCR fragments up to 350 bp of the plant-specific PEPCase gene of *Brassica napus* were detected by Hellebrand et al. [70]. Furthermore, Pauli et al. [95] showed that when crude soybean oil is simply

centrifuged at 14,000*g* for 15 min the level of DNA is reduced by at least a factor of 10^4, but DNA can still be detected. During the refining process, however, high temperatures, acidic pH, and adsorption by clay may reduce the DNA content of the oil [68]. Samples taken at various stages of the soybean oil-refining process showed that the degumming step, in particular, removed the DNA from the oil phase, rendering amplification in refined oil impossible [68, 94]. Similarly, Pauli et al. [54] could not detect the species-specific *zein* gene (277 bp) in refined corn oil.

Other processes

Irradiation

Extension of shelf-life and improvement of technological qualities are the objectives of radiation processing of foods. Villavicencio et al. [96] irradiated genetically modified soybeans up to 1000 Gy. DNA damage increased with increasing radiation doses, but GMOs could still be detected on the basis of screening of the 35S promoter of soybean samples. Higher irradiation doses of 10, 25, and 50 kGy were used on potatoes and on intermediate and final products of potato-stick production by Bauer et al. [73]. DNA seemed to be degraded by 10 kGy, because amplification of a 325-bp DNA fragment failed for some of the samples.

Sonication

Sonication was performed on DNA solutions by Debode et al. [116] and Shokere et al. [114]. Both showed on agarose gels that after 5 min DNA is degraded, but amplification by real-time PCR remains possible [114], even after 8 h [116]. The effectiveness of degradation by sonication decreased with time, however [116].

DNA quantification methods

In the framework of PCR-based GMO analysis, several methods have been used to evaluate the effect of food processing on DNA degradation. Agarose gel electrophoresis, spectrometry, conventional PCR, and real-time PCR (with assays of different length) are most widely used. In some cases nested PCR and quantitative competitive PCR have been applied.

Agarose gel electrophoresis

Agarose gel electrophoresis is based on separation of DNA fragments under the influence of an electric field, followed by staining of the DNA with ethidium bromide. For unprocessed samples, a tight band of undegraded and high-molecular-weight DNA can be observed. When DNA has been degraded or sheared during processing, the degraded DNA will be visualized as a smear of low(er) molecular weight DNA. Reduced intensity of a DNA smear can also be proof of DNA degradation. The method enables very rapid and quite sensitive estimation of DNA degradation, when several steps during food processing can be compared with a standard or with each other. However, in most cases comparison with a (series of) standards of known concentrations is not included. This makes comparison between experiments difficult, because evaluation of the result will depend on the quality of the gel staining and the picture taken and may be affected by the subjectivity of the analyst. Comparison would also be facilitated if clear definitions are used for high and low-molecular-weight DNA. In the context of food processing, the following different DNA weight fractions could be used: high (>20 kbp or unprocessed), medium (mainly 20–0.5 kbp), low (mainly 500–100 bp), and very low (<100 bp) molecular weight DNA. It should also be emphasized that the result of DNA analysis based on agarose gel electrophoresis is no indication of the subsequent result of PCR analysis. On one hand, PCR inhibitors may still be present, rendering DNA amplification difficult or impossible. On the other hand, a positive PCR amplification may be obtained even when no clear signal is observed, because, theoretically, only one target gene copy is needed for amplification. Nevertheless, agarose gel electrophoresis has been successfully used in comparison of the yield of different DNA extraction methods ("A" in Tables 1, 2 and 3). This method also gives a good indication of DNA degradation when processed and unprocessed samples are compared or when different samples are taken during processing and compared with each other (Tables 4, 5 and 6).

UV spectrophotometry and fluorescence measurements

Both UV spectrophotometry and fluorescence assays determine the concentration of DNA in a solution. The assays give a quick result and can be used to compare the yields of different extraction methods or the effect of DNA degradation in processed food samples compared with a DNA extract of the same sample before processing.

Spectrophotometric analysis uses UV absorption (A_{260}) for quantification of DNA molecules [4, 114]. The result indicates the total DNA content of the sample. However, accurate analysis will be affected by the purity (i.e. absence of proteins, RNA, phenol, …) and the amount of DNA. Usually, 2.5–5.0 μg DNA is needed for a measurement, and this might not be possible for some complex food matrices

[4, 142]. Spectrophotometric assays are also unable to differentiate between DNA and RNA and will not reflect DNA fragmentation. The accuracy and precision is further affected by the size distribution of the DNA in solution [4], so samples should be vortex mixed before measurement.

An alternative to UV spectrophotometry is DNA quantification by fluorescence spectrometry, because several fluorescent dyes interact with DNA. Again, accuracy will be affected by the size distribution of the solution. In cases of dyes which bind exclusively to double-stranded DNA (e.g. PicoGreen, Hoechst dyes and SYBRGreen), the single-stranded DNA or RNA present will not be determined [4, 114, 142]. Fluorimetric analysis also requires use of a DNA standard of comparable size [4]. Although fluorescence measurements are highly specific for double-stranded DNA and enable detection in the range 0.2–200 ng DNA, the result may be prone to errors depending on percentage AT, pH, and impurities such as salts and organic solvents [143].

Comparison of the spectrophotometric method with fluorescence assay using PicoGreen dye revealed that genomic maize NK603 DNA became degraded during heat treatment at 95 °C according to the fluorescent dye method whereas the concentration remained quasi-stable with the A_{260} method. Apparently, as DNA degradation increases, the quantity of DNA measured by the fluorescent dye method decreases, whereas the quantity measured by use of A_{260} increases slightly [114, 135]. Accurate measurement of the quantity of DNA in a solution is, however, of utmost importance in GMO analysis, because the result is the basis for calculation of copy numbers to determine the limit of detection of a method or the quantity of a specific gene (copy number). For processed foods, the fluorimetric method may therefore be preferred.

DNA quantity is however usually measured by UV spectrophotometry, in particular in evaluation of the yield of DNA extraction methods ("U" in Tables 1, 2 and 3) and in the evaluation of the effect of food processing on DNA degradation (Tables 4, 5 and 6). Only in a few cases is fluorescence used for comparison of DNA extraction methods ("F" in Tables 1, 2 and 3) and for DNA quantification during processing (Tables 4, 5 and 6).

Conventional PCR

Most studies on DNA degradation of GM food use conventional PCR as the main analytical tool. Conventional PCR is usually used as a qualitative assay (the target gene is absent or present). The PCR is therefore able to identify the processing step(s) which result(s) in the loss of a target molecule [75]. Different studies evaluating the effect of processing on DNA degradation are summarized in Table 4 for soy and soy products, and in Table 5 for maize and maize products. In the context of GMO analysis, conventional PCR has further been investigated in the processing of wheat and wheat bread [128, 133], the grinding of wheat [100], the refining of rapeseed oil [70], feed pelleting with canola [131] or wheat [65], potato processing [2, 73], tomato juice production [69, 108], sugar manufacture [141], and ethanol production from potatoes [137].

In the development of a PCR for evaluation of DNA degradation, the choice of the target gene is crucial. The use of multi copy number target DNA, e.g. chloroplast-specific amplicons, may result in a more sensitive assay, but the variation of the DNA quantity between cultivars may be higher because of variations in the numbers of copies of the DNA targets [142]. Preference should therefore be given to detection of single copy genes.

Experiments based on the use of different PCR assays which target the same gene but result in amplicons of different lengths have also been successfully used by several researchers to assess the effect of food processing on the degradation of plant DNA [2, 58, 69, 76, 79, 80, 83, 84, 100, 108, 112, 124, 128, 137]. Of course, the higher susceptibility to degradation of longer DNA fragments compared with shorter fragments should be taken into account.

Although conventional PCR methods are sensitive, the time of analysis is quite lengthy (compared with real-time PCR assays), resolution is quite low, the system cannot be automated, the dynamic range is rather small, and the post-contamination risk is higher than for real-time PCR. Furthermore, because it is an end-point measurement followed by agarose gel electrophoresis and, in most cases, visualization of the results with ethidium bromide, the intensity of the DNA band obtained cannot be related to the initial concentration of the target molecule.

Care should therefore be taken when analysing the results of conventional PCR analysis. Many different PCR assays may exist which target the same gene or DNA sequence. Between those assays, primer sequences, concentrations and types of reagents (e.g. *Taq* polymerase) may vary the number of cycles used might differ, and the length of the amplicon obtained and the DNA content in the PCR may differ from sample to sample (Tables 4 and 5). These factors will all have an effect on the sensitivity of the test. Moreover, in many of the cases studied for this review, information on the detection limit of the assays is lacking. Either the detection limit has not been tested, expression of the result varies (i.e. copy numbers, ng DNA, or GMO %) or reference is made to a PCR method described elsewhere but not tested during the research. The overviews in Tables 4 and 5 include detailed information regarding the target and target length, the number of PCR cycles, and the amount of DNA added to the PCR. Furthermore, information is added about whether food processing experiments have been evaluated with other DNA quantification methods.

In nested PCR, an outer and an inner pair of primers are used within the target region in two consecutive rounds of PCR amplification. As a consequence, the specificity of the test is higher and the detection limit lower than for conventional PCR. Because of the increased contamination risk associated with use of amplification products as template in the second PCR, the method has been used by a few researchers only for detection of (GM) food products [16, 38, 55, 70, 76, 92, 93, 98, 138, 144, 145] and for evaluation of DNA extraction methods [20, 39], but little research has been conducted on its use during GM food processing [95].

Quantitative competitive PCR (QC-PCR)

Competitive PCR is based on the amplification of two DNA sequences in the same test tube: an internal control and the target sequence of the sample under investigation. Both sequences are amplified, and, because of their different amplicon length, can be separated on an agarose gel. If the sample is amplified several times in different test tubes, containing increasing amounts of the competitor DNA, a semiquantitative measurement can be obtained when the intensity of the target DNA fragment is compared with that of the internal control. The equivalence point, where both targets have the same intensity, will determine the concentration of the sample under investigation. An advantage of competitive PCR is that PCR inhibitors have the same effect on both target and internal standard [146]. Competitive PCR is however only useful when the efficiency of both concurrent amplifications is the same. Other disadvantages are the low throughput, the visualisation of the result, and the use of agarose gel electrophoresis [146], which is time-consuming. QC-PCR has been developed for several GM target genes [132, 147–153]. Competitive PCR has been used during evaluation of sugar manufacture [141] and some heating processes (95 °C for 5, 10, 30, and 60 min) [132].

Real-time PCR

Real-time PCR is the method of choice for quantification of GMOs in food and feed (qPCR). The PCR amplification is followed kinetically with the aid of a fluorochrome, chemically linked to a probe. The number of PCR cycles necessary to generate a signal statistically significant above the noise or baseline signal is taken as a quantitative measure and is called the cycle threshold (Ct). The initial template concentration is determined on the basis of the PCR cycle at which fluorescence is first detected to be statistically significantly above background. Using a calibration curve derived from DNA samples of known concentration, the measured fluorescence is linked to the quantity of DNA in a sample. Its amount can be expressed in picogrammes or in number of copies, if the genome size is known [13, 154, 155].

The advantages of real-time PCR are the high sensitivity and specificity, the reproducibility of the results, the possibility of quantifying gene copies in a large dynamic range, and the straightforward design of a PCR procedure. A real-time PCR analysis is less labour-intensive and no post-PCR manipulations are required compared with conventional PCR. Compared with QC-PCR, real-time PCR is more sensitive and more accurate. Generally, real-time PCR assays are also directed at target molecules in the range 80–150 bp, which makes them very suitable for use in the analysis of processed foods, for evaluation of the effect of processing on DNA, and evaluation of DNA extraction efficiency. For detection of GMOs, both targets for the endogenous gene and the transgene should be of similar length in order to obtain similar PCR efficiencies for both targets. The kinetics of DNA degradation may also be studied using different primers which result in amplicons of different lengths in separate assays [29, 67, 73, 75], or in a single assay (multiplex PCR). The sensitivity of the real-time PCR assay may, however, be affected by the choice and concentrations of reaction mix constituents [156] and by the chemistry used. Several formats can be used:

1. the ds-DNA-binding dye SYBRGreen I [122, 157, 158];
2. hybridisation probes or fluorescence resonance energy transfer (FRET) probes [157];
3. hydrolysis probes, e.g. TaqMan technology [151, 158];
4. molecular beacons [159]; and
5. Scorpion probes [158].

When studying GM food processes TaqMan technology is usually used. Only in a few cases has the SYBRGreen method been used [67, 122].

Table 6 gives a summary of the use of real-time PCR for evaluation of DNA degradation in (GM) soy, maize, and potato products. Similarly to conventional PCR, the amount of DNA initially added to the PCR mixture may affect the quantification limits. Therefore, detection and quantification limits should be very well established. It has been estimated that, generally, ten target copies are required for reliable detection in real-time PCR, whereas at least 40 target copies are required for accurate quantification of that target using qPCR [27, 160, 161]. In some of the cases studied, an absolute number is given for the detection limits (copy numbers or picogramme of DNA) [29, 67, 68, 75, 114, 117], whereas in other cases relative limits (referring to the initial target copy numbers in the PCR) are used [80, 114]. Again, these limits are not always mentioned or have not been determined [72, 73, 122]. Furthermore, the choice

of, and the stability of, a specific target sequence may affect the test result. Comparison of four real-time PCR systems for detection of maize resulted in LOD values of 30 (*ivr1*), 10 (*zein*), 4 (*Adh1*), and 1 genome copy for the *hmga* systems. The LOQ ranged from 100 (*ivr1*) and 100 (*zein*) to 40 (*Adh1*) and 10 (*hmga*) maize genome copies [162]. Real-time PCR is also an efficient tool for evaluation of DNA extraction methods [28, 39, 51].

Conclusion

Many processing steps affect the state of the DNA present. High temperature and low pH are the most important factors which break down DNA. In many cases, however, PCR amplification will remain possible, irrespective of the matrix. The amplicon 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 not. 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 [123].

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