Polymerase chain reaction: a powerful analytical tool in the field of food safety

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Abstract

The polymerase chain reaction (PCR) is the canonical DNA analysis technique that has enabled the understanding of the complexity of organisms and significantly advanced achievements in the field of biological sciences. Today, the method is still widely used in basic science research, but PCR-based diagnostics are becoming increasingly important in industries such as food and agriculture. This method provides high sensitivity because it requires trace amounts of template DNA to generate enough copies for detection. Using primers specific to the DNA sequence ensures the high specificity of the test. The advantages of the method are time – and cost-effectiveness and reproducibility. PCR-based techniques have found favor in areas where food traceability is important, whether from an economic, legal, health, or religious-cultural point of view. This review outlines the most important aspects in which the PCR method has been successfully applied, namely in the protection of human health by enabling the identification of foodborne pathogens or allergens. Furthermore, the use of PCR in the so-called green criminology, a branch that deals with tracking illegal practices such as food adulteration, compliance with the labeling rules, and detection of food products containing GMO material or other undeclared food ingredients, was also described.

Keywords: polymerase chain reaction, food adulteration, GMO, allergens, foodborne pathogens

Introduction

Polymerase Chain Reaction was invented by an American biochemist Kary Mullis an employee of the biotechnology company Cetus Corporation. The principle of the PCR method was published for the first time in 1986 (Mullis et al. 1986). For his discovery, the author received the Nobel Prize in Chemistry in 1993. The essence of the PCR reaction is to synthesize deoxyribonucleic acid (DNA) in vitro in laboratory conditions, which normally occurs in vivo, in living cells (Van Pelt-Verkuil et al. 2008). The reaction requires the presence of single or double-stranded DNA, synthetic primers designed specifically for the DNA region of interest, deoxyribonucleoside triphosphates ($_{d}$NTPs), the appropriate concentration of magnesium ions (Mg^{2+}), and the presence of a DNA polymerase enzyme. *Taq DNA Polymerase* is a thermostable enzyme originally isolated from the eubacterial microorganism *Thermus aquaticus* and is widely applied due to its notable high-temperature resistance, high specificity and sensitivity, high efficiency, and reproducibility. Amplification is performed by thermal cycling, a process in which the reaction is repeatedly heated and cooled to produce multiple copies of the target DNA region. The entire process of synthesizing a new DNA strand complementary to the template DNA consists of three main stages. The process is initiated by the denaturation step in which a high temperature (95 °C approximately) breaks hydrogen bonds of the double-stranded DNA ($_{ds}$DNA) and separates it into two single strands. This allows primers to access single-stranded DNA ($_{ss}$DNA) and then select and bind (annealing step) to a complementary sequence on the $_{ss}$DNA template molecule. The annealing step is performed at a lower temperature of approximately 50 °C, and its exact temperature depends on the primer‘s length and sequence. In general, the annealing temperature should be approximately 5 °C lower than the melting temperature (Tm) of the primers used. Subsequently, the ssDNA/primers mixture is heated to 72 °C

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and the extension stage begins in the presence of a thermostable polymerase, $_{d}$NTPs, and Mg^{2+} molecules. In this way, a new double-stranded DNA molecule is created from each single strand of the original DNA template. These three steps are repeated approximately 40 times, each time resulting in a doubling of the DNA copy number. The typical PCR temperature profile is demonstrated in Table 1.

Table 1. Polymerase chain reaction program steps and conditions

Step	Time	Temperature	Comments
Initial PCR activation step	15 minutes	95 °C	The time can be reduced to 5 minutes if the reaction mastermix does not contain HotStart polymerase
		40 cycles of:	
Denaturation	30 seconds	95 °C	Double strand DNA denaturation
Annealing	30 seconds	50 °C	Primers annealing, the temperature depends on primers melting temperature
Extension	30 seconds	72 °C	Elongation of the target sequence
		End of repeating cycles	
Final extension	5 minutes	72 °C	Final target sequence extension

The complete PCR process provides a million copies of the original DNA fragment. The end product of the amplification can be evaluated in terms of quantity and fragment size using agarose electrophoresis. Moreover, PCR is often used to amplify DNA for sequencing or other downstream applications. The variations based on PCR principles that are commonly used today and give a more accurate result are Real-Time PCR methods (RT-PCR). In this technique, the increasing number of DNA copies during thermal cycling can be monitored using a CCD camera. The amount of DNA accumulated in each cycle is determined by monitoring the increase in ethidium bromide fluorescence binding to the copied DNA (Higuchi et al. 1993). The performance of Real-Time PCR methods depends on the nature of the chemicals that make up the amplification reactions. Currently, two types of fluorescent reporters are widely used; SYBR green and *Taqman* probes. Unlike conventional PCR, which requires visualization of the final product on an agarose gel, RT-PCR allows monitoring of the increase in the amount of target DNA during the reaction (in real-time). The recognition that DNA fragments of any length can be amplified using fragment-specific primer pairs in a single reaction become an important milestone in the development of diagnostics, which continues to rely largely on the PCR method. Initially, this method was developed as a tool for disease diagnosis, and its first application was the detection of mutations in the *β-hemoglobin* (HBB) gene, which is responsible for the development of sickle cell anemia. Very recently, during the COVID-19 pandemic, many variants of PCR-based diagnostic tests have been applied to *Coronavirus* detection (Jayakody et al. 2022; Van Kasteren et al. 2020), which confirms that PCR remains the gold standard in diagnostics so far. There is no doubt that the invention of the PCR method significantly influenced the increase in research achievements in different fields of biological sciences. Currently, the PCR technique is used in many areas, from clinical and veterinary analyses to environmental analyses, criminal investigations to food analyses.

Genetically modified organism identification

The advent of genetic engineering techniques allows the transfer of genetic material across completely unrelated species. The first genetically modified food crop approved

for commercialization was the Flavr Savr™ tomato in 1994, intending to slow down its ripening and rotting process (Kramer a Redenbaugh 1994). Since then, 190.4 million hectares of planted GM plants in 29 countries were reported in 2019 (ISAAA 2023). The ambiguous approaches of consumers towards products containing GMO material revealed the need to track these food products. Thus, according to European Union regulations, every food product containing material consisting of or produced from GMOs in a proportion greater than 0.9 % is subject to labeling (Castellari et al. 2018). The traceability of GMOs in the food/feed chain is a key factor in complying with these regulations. Currently, there are two main types of transgenes detection in use, categorized as indirect (protein-based) and direct (DNA-based) methods. Protein-targeted methods are often used to screen GMOs, but their suitability for processed food products often fails due to the degradation of the target molecules. On the other hand, the direct method provides high sensitivity and specificity in detecting DNA sequences even in difficult biological material, therefore the PCR-based techniques represent the greatest common denominator in global analyses for the identification and quantification of GMOs. Genetically modified plants developed for agricultural purposes contain a recombinant DNA construct, the so-called transgenic cassette that contains certain regulatory elements common to all transgenic plants. Typically, transgenic cassettes consist of elements from non-host species and contain the desired gene sequence (trace gene), the expression of which is regulated by an upstream promoter and stabilized by a downstream terminator (Dugdale et al. 2014). Typically, GMO tracking involves detecting the DNA sequences of these three regulatory elements during the screening phase of GMO analysis. The schematic illustration of the transformation proces and GMO detection methods based on genetic analysis is presented in (Plate I, Figure 1)

The most commonly used promoter sequence is CaMV35S from the *Cauliflower mosaic virus* (abbreviated p35S) and the NOS terminator from *Agrobacterium tumefaciens* (abbreviated tNOS). For this reason, p35S and tNOS are the most frequently analyzed sequences when confirmation of the presence of a transgene construct is required. Another possible approach is the detection of an inserted gene that carries a new desired plant characteristic. GMO modifications have been introduced in most popular crops. Different PCR-based methods were developed to detect GMO material in cotton (Randhawa et al. 2013), maize (Mano et al. 2010), papaya (Yamaguchi et al. 2006), pepper, tomato, eggplant, potato (Chaouachi et al. 2008), rapeseed (*canola*) (Lee et al. 2021), rice (Safaei et al. 2019), soya bean (Park et al. 2021), squash (Green et al. 2004), sugar beet (Chaouachi et al. 2013), watermelon (Bi et al. 2019) and wheat (Terzi et al. 2003). European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) authorized few PCR assays for EU legal purposes (European Commission, GMO, 2023).

Allergen analysis

Food allergies are a serious global health problem that affects people of all ages. Current data indicate that food allergy prevalence has been increasing in the last 30 years and affects approximately 1-10 % of the general population (Gupta et al. 2023). Food allergy can be defined as the hypersensitivity of the body to a specific substance present in a food that triggers an excessive immunological reaction. The substance that causes an autoimmune response is an antigen – a protein whose presence in the body causes the production of antibodies that trigger an allergic reaction. Therefore, the antigen that causes allergic reactions is called an allergen. An allergic reaction caused by food can cause adverse symptoms such as fatigue, weakness, nausea, and vomiting, and may range from skin rash to anaphylactic shock. Most reactions cause mild symptoms, but some

are severe and may even be life-threatening. Although the sources of food allergens are well known, the presence of undeclared allergenic ingredients or the presence of traces of allergens as a result of contamination during food processing poses enormous health risks to sensitized individuals. Food businesses are obligated to provide safe food, which means declaring all allergens present in ingredients and warning consumers about their potential contamination. Therefore, there is a great demand for reliable analytical methods for identifying allergenic substances in food products. The immunological methods including enzyme-linked immunosorbent assay (ELISA) are well-established in allergen detection and are still considered as the gold standard (Linacero a Cuadrado 2022). The ELISA test directly reacts with food allergens and is a cost – and time-effective method, which certainly constitutes the advantages of this method. On the other hand, the disadvantage of ELISA is its low sensitivity in processed food products due to protein denaturation and the possibility of crossreactions that lead to under- or over-estimation of the target analyte (Jiang et al. 2021). Therefore, PCR-based methods can be a reliable alternative to ELISA because DNA is a more stable molecule than protein. In PCR analysis, the target sequence is the sequence of the gene encoding an allergy-related protein or a specific gene sequence from food containing this protein (López-Calleja et al. 2013; Suh et al. 2019). It is worth emphasizing that in the PCR test, it is possible to use several pairs of primers to detect multiple targets in a single reaction (so-called multiplex PCR), thus reducing costs and time. The PCR method was successfully implemented for the detection of gliadin which is a major component of wheat gluten and is responsible for most of the harmful effects on the health of sensitive people (Sánchez-León et al. 2021). PCR method using a pair of primers specific for the gluten-coding DNA sequence revealed the presence of gluten in guaranteed gluten-free products but also in naturally glutenfree foods. These were mainly gluten-free sponge cakes, gluten-free biscuits "Cranberries", cocoa powder, coffee "3in1", and instant coffee (Výrostková et al. 2022). *García-García and colleagues* (García-García et al. 2019) used primers specific for a DNA fragment on the ribosomal internal transcribed spacer (ITS) as a genetic marker to track gluten in 220 food products. The *ribosomal internal transcribed spacer* (ITS) region is common for three main gluten-containing cereals, namely wheat, barley, and rye. The developed PCR methodology was used to screen gluten-containing cereals in 220 food products available on the market. Hazelnuts (*Corylus avellana*) and peanuts (*Arachis hypogaea*) are well-known allergens present in pastries, confectionery ice cream etc. Its undeclared amounts may occur as a result of unintentional cross-contact with other foods. Engler-Blum et al. developed multiplex PCR which has a practical detection limit of 10 mg/kg for both peanuts and hazelnuts with any cross-reactivities (Engler-Blum et al. 2007). Compared to the ELISA technique, PCR-based methods show better performance for the detection of traces of celery (*Apium graveolens*) in food matrices targeting the gene coding mannitol dehydrogenase (Hupferet al. 2010) or NADPH (*Nicotinamide Adenine Dinucleotide Phosphate*)-dependent mannose-6-phosphate reductase (Fuchs et al. 2013). Currently, there are commercial PCR-based kits available on the market for determining the presence of trace amounts of celery in food products (SureFood® Celery with a limit of the detection of 0.4 mg/kg). PCR shows better performance in the case of fish allergen detection as ELISA tests struggle with high cross-reactivity and low sensitivity (Dong a Raghavan 2022). The multiplex PCR method enables the detection of tropomyosin sequences from oysters (*Crassostrea gigas*), mussels (*Mytilus edulis*), clams (*Ruditapes philippinarum*), and abalone (*Haliotis discus hannai*). This method was applied to processed products, frozen and dried seafood as well as seasoned, porridge, and canned seafood products (Suh et al. 2020). The PCR method has been widely used in the detection of food allergens, in some cases, such as the detection of trace amounts of celery or fish, it is irreplaceable. However, PCR does have its limits: the detection of eggs or milk is difficult using PCR since the analysis would only detect

chicken or cow DNA. Moreover, food products such as vegetable oils, gelatin, lecithin, egg white, and yolk or starch contain just single copies or no DNA and are a challenge for PCR tests (Grundy et al. 2023).

Foodborne pathogen analysis

According to the World Health Organization (WHO), unsafe food causes 600 million cases of diseases and 420,000 deaths worldwide each year and also has serious economic consequences (WHO 2023). Pathogens such as bacteria, viruses, fungi, parasites, and yeasts are the most common food contaminants and are responsible for foodborne illnesses. Contamination can occur at any stage of food production, from growth, through harvesting, processing, storage, transportation and preservation, and finally food consumption. Foodborne pathogen detection is intended to ensure compliance with regulations that set maximum levels of certain pathogens in certain categories of food products to prevent harmful products from entering the consumer market. Furthermore, it is essential to identify and control the spread of pathogens before a serious outbreak occurs. The conventional culture-based method has been historically established as the „gold standard" for identifying foodborne pathogens. Although this method is inexpensive and simple to perform, the methodology is time-consuming and requires waiting 2-3 days for the results (Wiemer et al. 2011). Additionally, false-negative results occur often, so this method requires complementation with other methods, such as PCR, to ensure that the desired pathogen has been isolated (Foddai a Grant 2020; Law et al. 2015). Moreover, unlike multiplex PCR, the culture-based method is unable to detect multiple pathogens in parallel. Molecular biology techniques, especially PCR, are becoming an increasingly popular alternative to conventional methods due to their high diagnostic effectiveness. For example, Liu and colleagues successfully developed a high-throughput real-time PCR assay capable of simultaneous detection of 12 of the most common foodborne bacterial pathogens, namely *E. coli, L. monocytogenes/ivanovii, S. enterica, V. parahaemolyticus, β-streptococcus hemolyticus, Y. enterocolitica, E. faecalis, Shigella spp., P. mirabilis, V. fluvialis, S. aureus,* and *C. jejuni.* Interestingly, the new method allowed analysis time to be reduced to ~1 hour compared to the 7 or more days required for traditional culture-based methods (Liu et al. 2019). Different PCR-based methods such as endpoint PCR, real-time PCR, nested PCR or loop-mediated isothermal amplification (LAMP) have been applied for the detection of mycotoxigenic fungi in complex food and feed matrices (Rahman et al. 2020). Multiplex PCR revealed high performance in the simultaneous detection of *Aspergillus, Penicillium*, and *Fusarium* in contaminated maize grain powder (Rahman et al. 2020). The PCR method with specific primers was able to distinguish aflatoxin-producing fungi from non-aflatoxin-producing fungi in "meju", a Korean fermented soybean food (Kim et al. 2011). Quantitative PCR followed Reverse Transcription Quantitative PCR followed Reverse Transcription (RT-qPCR) is a useful method, especially for the analysis of viral food pathogens since a significant part of viruses are RNA viruses. This method allowed to detection of *Hepatitis A* and *Norovirus* in mussels (La Bella et al. 2017), and soft berries such as raspberries, strawberries currants blueberries blackberries, and blackcurrants (Fraisse et al. 2017), or lettuce (Coudray-Meunier et al. 2015). Moreover, *Rotaviruses* and *Adenoviruses* were detected with high sensitivity and specificity in beef, poultry, and pork meat using the RT-qPCR method (Soares et al. 2022).

Food adulteration analysis

Food adulteration means intentional interference with the composition of a food product by adding or replacing a food substance with an undeclared substitute or removing

a valuable ingredient characteristic of this food product. This is usually intended to maximize the manufacturer‘s profit by reducing production costs and increasing the bulk of a given product. Food falsification also refers to giving misleading names to products and introducing false information regarding their composition, production method, or origin. This is a non-trivial problem for consumers because it is often inappropriate from an economic, health, religious, or legal point of view. To fight this illegal practice, various laboratory techniques for identifying food falsification have been developed. Sensory and morphological characteristics such as color, taste, smell, and shape were found to be unreliable, as was physical analysis. Advanced methods based on lipids and protein analysis often fail due to the instability of these molecules during food processing. Currently, the most useful tests for detecting food adulteration are based on DNA detection, especially PCR-based methods, because they are very sensitive and specific, cost – and time-saving (Haji et al. 2023). For instance, almonds (*Prunus dulcis*) that are used to produce marzipan are often replaced with cheaper alternatives such as persipan. Persipan is a less expensive alternative to marzipan and is produced from apricot (*Prunus armeniaca*) or peach kernel (*Prunus persica*) and both show great similarity in texture and appearance to marzipan. The TaqMan qPCR method developed by Gansbeke and collective (Van Gansbeke et al. 2018) revealed high specificity for the apricot kernel. The method was able to detect as low as 1 % persipan in marzipan. Commercial saffron obtained from dried stigmas of *Crocus sativus L.* is the most expensive spice on the global market and is classified as one of the most frequently adulterated foods. Safflower (*Carthamus tinctorius L.*) due to its deep red color of carthamin is often used for saffron adulteration (Petrakis et al. 2015). Villa et al. (Villa et al. 2017) proposed species-specific RT-PCR with primers targeting the ITS region of safflower. This assay allowed to detection of 0.1 % of safflower in saffron. The optimized method was further applied to commercial saffron samples such as stigmas, powders, and seasonings. Another study showed that RT-PCR is a suitable method for detecting adulterated honey (Sobrino-Gregorio et al. 2019). In this study, the presence of rice molasses in adulterated honey has been successfully detected. The method was highly sensitive and allowed the differentiation of different amounts of rice molasses in honey. In recent years, the number of consumers adopting a vegan/vegetarian lifestyle has been increasing. As a result, the demand for meat-free products is growing exponentially, which has resulted in many new legal regulations requiring tools to control compliance with them. For example, the European V label specifies in its guidelines that the total contamination with animal residues in the final product must not exceed 0.1 % (1 g/kg) (European Vegetarian Union. Criteria of the V-label Regulations, 2023). The usefulness of the PCR method in this area is sufficiently established, which is confirmed by the presence of commercial kits for analyzing trace amounts of animal DNA in vegan products. Further, Köppel and colleagues (Köppel et al. 2021) designed a multiplex PCR that enables simultaneous detection of animal fish and plant DNA in food samples. The detection limit they achieved was even lower than recommended by the European labeling and amounted to 0.032 %. Moreover, accurate PCRbased testing has shown high accuracy in identifying meat species such as porcine (Kang et al. 2021), which is important for meeting religious requirements for many food products.

Conclusion

Molecular techniques have gained recognition as a quality assurance tool in the food industry. This review briefly presents the possible applications of polymerase chain reaction in food safety, considering all branches of this concept. The area of interest includes aspects related to human health, such as identifying trace amounts of allergens or pathogens, as well as tracking illegal food adulteration and detecting traces of GMOs in food products. PCR-based methods provide results with a high level of sensitivity and specificity for the identification of DNA sequences in various food products. These methods are also time and cost-efficient and ensure repeatability. In summary, these results suggest that biomolecular tools may permanently find use as a stand-alone tool or alongside physicochemical, chemical, and biochemical methods.

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