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In food safety control overview of using Real-Time PCR

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Abstract

The identity-determining importance of nucleic acids in living beings is a guide for reaching the desired information about food in quality control analyzes. With these goals, the popularity of Real-time PCR (Polymerase Chain Reaction) methods, which are one of the nucleic acid-based methods, is rapidly increasing due to their high reproducibility, precision and fast result production. It is thought that informative studies about the analysis using the device will provide a basis for researches on the subject. This review contains information about the studies conducted on Real-Time PCR analysis used to detect imitation/adulteration and cheating in foods. General descriptions about the operation of the Real-Time PCR methods are given. The quality control analyzes in which the method was used were classified and explanations were made about each analysis area and examples from the studies in the literature were given.

Introduction

Food safety is a concept that must be followed from the first steps of the production process to reaching the end consumer. Whether the food has its characteristics, the production method, the reliability of the place where it is produced, regional differences, and the composition of the food should be known in terms of food safety. In parallel with the development of food production technologies, new techniques continue to develop in manipulations such as cheating, imitation, and adulteration. It is important that such deceptive practices can be detected before the food reaches the end consumer. Due to the variety of these deceptive applications, the analysis methods to be used in their detection show similar diversity. Spectroscopic, isotopic, chromatographic, immunological, electrophoretic, electronic, and nucleic acid-based analysis can be shown as examples of these methods (Yeşilören & Ekşi, 2014).

As a result of advances in molecular biology and biotechnology, Polymerase Chain Reaction (PCR) and fast and quantitative Real-Time PCR techniques have been used in food quality control analysis (Kesmen et al., 2017; Böhme et al., 2019).

PCR and real-time PCR

Mullis et al. (1986) described the polymerase chain reaction (PCR) in 1986 and won the Nobel Prize in Chemistry in 1990. PCR methods have been used in many areas from clinical analysis to environmental analysis, from criminal tests to food analysis (The Nobel Prize Web Site, 1993; Mullis et al., 1986). The high sensitivity and reliability in the Covid-19 (Corona Virus) epidemic that started to spread from Wuhan City of China in late 2019, the method described by Corman et al. (2020) made the Real-Time PCR method very popular.

The purpose of PCR reactions is to perform the synthesis of Deoxyribonucleic acid (DNA) *in vitro*, which takes place *in vivo*. The reaction takes place in the

presence of single or double DNA strands and oligonucleotide primers and deoxyribonucleoside triphosphates (dNTP) in the presence of a heat-resistant polymerase enzyme and magnesium ions. During the reaction, in reactions where the Taq DNA polymerase enzyme is used, the temperature is raised and lowered between high temperature (95°C) to separate the double-stranded DNA strand into a single strand, and then between the binding temperature (72°C) for the primers in the environment to complete the chain with dNTPs (Kubista et al., 2006; Holland et al., 1991).

At high temperature, breaking of double stranded DNA to single stranded is called denaturation, and elongation of primers with nucleotides with the appropriate DNA polymerase enzyme in the presence of Mg^{2+} ions is called primer extension (Kubista et al., 2006; JRC European Commission, 2006).

Taq DNA Polymerase enzyme produced from *Thermus aquaticus* is widely used due to its high-temperature resistance, high specificity, efficiency, sensitivity, and reproducibility (Holland et al., 1991). The optimum operating temperature of Taq DNA polymerase is 72°C and most PCR protocols use this value as the extension temperature (Kubista et al., 2006). A buffer solution is also needed for PCR. This buffer also contains $MgCl^2$ solution at a concentration between 0.5 and 5 mM. Mg^{2+} ions form a dissolvable mixture with dNTPs, which increases polymerase enzyme activity and primer/target DNA interaction. Free dNTPs are required for DNA replication. It is preferred that the dNTP concentration is in the range of 20-200 µM. To prevent an incorrect match, 4 dNTPs must be used equally.

The design of the primers is critical to increasing PCR success. Even in an environment where all other factors are suitable, an incorrectly designed primer may cause the reaction not to occur. When designing the primer, attention should be paid to primer length, melting temperature, sensitivity, complementary sequences, Guanine/Cytosine (G/C) ratio, polypyrimidine (Thymine and Cytosine) and polypurine (Adenine and Guanine) elongations, and 3' end sequence characteristics (JRC European Commission, 2006; Innis & Gelfand, 1990).

The PCR methods that are widely used today and give the most reliable results are Real-Time PCR methods. Higuchi et al. (1993) designed the PCR technique in which the increase in DNA amount during thermal cycles can be monitored with the help of a CCD camera. In this technique, the amount of DNA accumulated in each PCR could be determined by monitoring the increase in ethidium bromide fluorescence binding to the copied DNA (Higuchi et al., 1993). This study formed the basis of Real-Time PCR. The performance of Real-Time PCR methods depends on the nature of the chemicals that make up the amplification reactions and the device used to maintain and monitor the reaction. Indicator dyes (ethidium bromide, SYBR Green I), hybridization probes (TaqMan probes, FRET probes, molecular beacons, scorpions, etc.) are examples of these factors (JRC European Commission, 2006). Unlike the end product analysis in conventional PCR, the increase in the targeted DNA (amplification) can be observed in the Real-Time PCR method during the reaction. In the Real-Time PCR method, probes and dyes that provide a signal depending on the number of DNA chains that increase during thermal cycles are used. While the signal is weak during the first cycles of the reaction, it rises logarithmically with the increasing number of DNA, and the signal reaches its peak at the end of the reaction. The first starting point of the fluorescent signal is related to the amount of target DNA at the beginning of the reaction (JRC European Commission, 2006; Ahmed, 2002; Zhang et al., 2019).

While the Real-Time PCR method is executed, the fluorescence value is plotted against the number of cycles (Cycle) on a semi-logarithmic scale. It is possible to follow 3 stages in this graph: The lag stage where slight signal fluctuations are seen, the logarithmic phase where increasing fluorescence data is collected and the plateau phase in which the signal value reaches a plateau. When measuring with the Real-Time PCR method, the logarithmic phase in which a rapid increase is observed rather than the signal value in the plateau stage is taken as a basis. The target gene content of the reacted product is the most important factor determining the cycle point where the logarithmic increase begins. This point is called the cycle threshold (Kubista et al., 2006; JRC European Commission, 2006).

Genetically modified organism (GMO) analysis

The rapid developments in gene technology in recent years have made it easy and efficient to change the genetic structure of plant products with commercial concerns. In agricultural production, the expectations to increase the amount and quality of the product resulted in the increased production of genetically modified products that from 1.7 million hectares to 190.4 million hectares from 1997 to 2019 (International Service for the Acquisition of Agri-biotech Applications (ISAAA), 2018). Different approaches of consumers to products containing GMOs has revealed the need to identify foods containing these products. Analysis methods used to identify GMO products can be examined under two main headings, protein-based and DNA-based. Protein-based analysis methods can be listed as the "immunoassay" method, "Western Blot" method, "ELISA" method, and "Lateral Flow Strip" method. DNA-based analysis methods are the "Southern Blot" method and PCR/Real-Time PCR methods (Zel et al., 2012; Fagan, 2004).

In GMO analyses with Real-Time PCR, the presence of regulatory trace genes such as 35S promoter (CaMV 35S) and nos terminator and/or directly the presence of genetically modified crops such as MON87701 Soy, GHB614 Cotton, DAS 40278-9 Maize are investigated. Currently, as new Genetically Modified (GM) types are produced, analysis of these types can be made in the

Real-Time PCR method (Hernandes et al., 2010). There are commercially prepared analysis kits for the detection of these genes, as well as individual primer and probe designs for each gene, and analyzes can be carried out (JRC European Commission, 2006). Suitable forward, reverse and probe sequences for the CaMV 35S promoter are given in the table (Table 1).

Table 1. Primer and Probe Designs for CaMV 35S Promoter Gene (Alary et al., 2002)

Orientation	Sequence (5' → 3')
Forward Primer	CGTCTTCAAAGCAAGTGGATTG
Rewerse Primer	TCTTGCGAAGGATAGTGGGATT
Forward Probe	TCTCCACTGACGTAAGGGATGACGCA

Real-time PCR method allows looking at different gene regions in the same analysis as single, double, triple, and multiple. In this sense, the study of Samson et al. (2012) can be given as an example. In the study, Genetically Modified (GM) maize coded MON810 and GA21 were analyzed. In multiple analyses, detection and identification limit studies were made, and by creating a calibration table, GM corn in the products could be determined as a percentage (Samson et al., 2012).

While more than one gene region can be scanned in the same reaction well in multiple analyzes, single analyzes can also be used to look at more than one gene region in a single reaction using the same plate. Unlike multiple analyses, in a single analysis, the master mix has to be prepared separately for each gene region. This means extra time and labor loss (Alary et al., 2002). In the study of Gerdes et al. (2012), the products coded as NK603, Bt176, MON 810, TC1507, 59122, T25, GA21, Bt11, MON 863, MIR604, 3272, CBH-351, and 40-3-2 GM Soybean were analyzed. In the study where 2 parallel analyzes were performed, 72 wells were filled with positive and negative controls (Gerdes et al., 2012).

Meat species analysis

Meat has an important place in nutrition because it is a high and quality source of protein. It is heavily consumed by people for a balanced diet. While consumers prefer meat products, they benefit from some features such as religious beliefs, usefulness, and allergenic effects. Unlike the animals specified on the label of the product, producers can deceive consumers by using cheap meats in their products. For these reasons, knowing the animal that is source of meat product is very important for food safety (Kaya et al., 2019).

In the analysis of species determination in meat products, protein and DNA-based methods are used depending on the equipment, method, sample preparation costs, and form. Protein-based methods are Electrophoresis, Chromatography, Spectroscopy, and ELISA. DNA-based methods can be listed as Restriction Fragment Length Polymorphism PCR (RFLP-PCR), Multiplex PCR, Real-Time PCR, and Digital PCR. Real-time PCR methods are preferred in routine analysis because they can be applied to mixed meat products, their cost is lower than other DNA-based methods, and they give fast and precise results. It has been reported in the literature that single-copy genes or repeat sequences in the genome are used for animal species and generality in quantification studies. Quantification studies can be carried out by RT-PCR by targeting Mitochondrial tRNA, Mitochondrial rRNA, Mitochondrial cytochrome b, Transferrin, Myostatin, and Mitochondrial D-loop sequences (Alikord et al., 2018; Kaya et al., 2019).

There are many studies in the literature using Real-Time PCR on meat products. Iwobi et al. (2015) conducted a study determining the proportions of beef and pork in mixed minced meat samples. Iwobi et al. (2017) also carried out a study determining the mixing ratios of horse meat in meat products. Thanakiatkrai et al. (2019) performed gene screening for dogs, ducks, buffalo, goats, and sheep in Asian Food Products. Ali et al. (2012) performed pig gene screening in commercially produced hamburger patties. Fang and Zhang, (2016), did mouse gene screening in meat products; while Pegels et al. (Pegels et al., 2015) performed horse gene screening in food and feed samples sold on the Spanish Market.

Allergen analysis

Allergy can be defined as the body's hypersensitivity to the allergen. When explaining the term allergen, it is necessary to mention the antigen. The antigen is the name given to substances that cause antibody production if they enter an organism. If the antigen taken into the body produces antibodies that cause allergic reactions, this antigen is called an allergen. In other words, if the antigen taken into the body activates the body's immunological system against it, this antigen is called an allergen (Karakılıç et al., 2014; Tayfur & Ünlüoğlu, 1996). Allergic reactions caused by food can start from symptoms such as fatigue, weakness, drowsiness, nausea, vomiting, and can reach urticaria and anaphylactic shock (Tayfur & Ünlüoğlu, 1996).

Food allergens appear in a huge range of products. Examples of these products are peanuts, nuts, eggs, cow's milk, soybeans, fish, shellfish, bananas, chicken, chocolate, and wheat. The excess of allergen foods has led to the development of many analysis methods such as ELISA, PCR/Real-Time PCR, and Liquid Chromatography/mass spectrometry combinations to detect them (Tayfur & Ünlüoğlu, 1996; Kizis, 2014). In Real-Time PCR methods, the goal is to determine the specific gene sequence of the food ingredient that causes the allergy. Compared to conventional PCR, it eliminates the need for additional chemical materials such as gel electrophoresis, requires less sample compared to protein-based methods, is more closed to contamination across-contamination and provides high automation in large-scale enterprises, making Real-Time PCR more preferred in allergen analysis. TaqMan probe

methods are used in allergen tests. The target gene sequence to be used in the analysis is derived from the gene sequence encoding the protein related to allergy or a specific gene sequence of the food containing that protein (López-Calleja et al., 2013; Garber et al., 2016). Primers and probes containing these gene sequences can be designed commercially or studies can be performed using analysis kits. Herrero et al. (2014) detected fish-borne allergens in foods containing fish with the help of commercial kits. In another study, Herrero et al. (2012) designed a rapid Real-Time PCR method that can detect allergens in shellfish such as shrimp, crab, lobster and crayfish in a 40-minute analysis time. López-Calleja et al. (2013) devised a method that could detect peanut allergen by Real-Time PCR in processed foods. Primers used in the study were commercially designed, and analysis kit was not used.

Milk origin analysis

Milk and dairy products have an important place in nutrition. It contains a balanced composition to meet nutritional elements such as milk, protein, fat, carbohydrate, minerals, and vitamins. The need to know the animal species from which the food consumed is produced has increased emergence of different research areas in this regard. Labels of dairy products offered for consumption in the market include animal species information. To prevent misinformation and/or fraudulent production, effective analysis management should be used in origin detection (Kara & Demirel, 2016; Narayan Jha et al., 2016).

The method used to detect especially bovine proteins in dairy products works on the isoelectronic basis of ɣ-caseins after plasmolysis. This technique is used for cow's milk but may give erroneous results in heat-treated products and the presence of low contamination. To differentiate between buffalo milk and cow's milk, 13C nuclear magnetic resonance on triacylglycerols, an HPLC technique for β-lactoglobulin analysis on buffalo milk and mozzarella cheese, multivariate regression analysis and capillary electrophoresis and acrylamide gel technique are used. More recently, ELISA methods have been used. However, the negative features of these methods are that they are not suitable for routine use, take a long time, and do not provide absolute certainty (Di Pinto et al., 2017; Bottero et al., 2003; Lopparelli et al., 2007).

Analyzes using Real-Time PCR methods have been extensively applied in numerous tests to identify animal species. These methods are based on the persistence of genomic DNA extracted from somatic cells in both milk and cheese without degradation even in the applied manufacturing processes. The most common assays for species identification are based on PCR analysis of species-specific mitochondrial DNA (mtDNA) sequences. MtDNA can be considered an augmented genetic resource. Apart from these, there are well-known analytical methods for origin identification of dairy products based on nuclear DNA sequences such as β-casein genes and 18S - 28S rRNA multiple copy genes. In Real-Time PCR-based animal origin analyses, mtDNA is preferred because it contains more genes specific to the source and contains more gene sequences compared to nuclear DNA. In addition to Real-Time PCR methods based on the detection of a single species, multiple Real-Time PCR methods that can detect more than one species at the same time provide faster and more economical origin determination (Bottero et al., 2003; Lopparelli et al., 2007; Xue et al., 2017; Kotowicz et al., 2007).

Agrimonti et al. (2015) conducted a study in 2015 on a rapid method design that can detect cow, goat, sheep, and buffalo genes in dairy products with Real-Time PCR. They also developed a method for determining the proportion of cow's milk in products using milk mixtures. Lopez-Calleja et al. (2007) studied a method for determining the proportion of goat milk in sheep milk using a Real-Time PCR method. By comparing the CT values obtained in the reactions, they determined the percentage ratios in the mixed milk. Lopparelli et al. (2007), investigated the determination of cow's milk mixing ratio in buffalo mozzarella cheese using the Real-Time PCR method. In the study conducted on 64 cheese samples, it was determined that the most of the products were contaminated with cow's milk. Kara and Demirel (2016), determined the origin of milk used in the production of Afyon Cream by Real-Time PCR method. In the study, 100 cream samples were analyzed and as a result, it was determined that 13% of these products were produced from buffalo milk, 28% from a mixture of buffalo milk, and 59% from cow milk.

Microbial pathogen analysis

Microbial diseases caused by food are important health problems caused by the consumption of foods contaminated by pathogenic microorganisms. At any stage from the production of food to consume, pathogenic microorganisms that contaminate food cause serious disturbances in the people who consume them. When pathogenic microorganisms such as *Listeria monocytogenes*, *Campylobacter*, *Escherichia coli* O157: H7, and *Salmonella* enter the body with the foods they contaminate, the metabolic products of these microorganisms cause infections. Bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridium botulinum* can produce toxins. Infections also occur by taking these toxins into the body (Tutar et al., 2015; Rathnayaka et al., 2018; Halkman, 2019).

The methods used to identify pathogenic microorganisms can be listed as colony-based methods, immunological methods, and molecular biological methods. Colony-based methods have disadvantages such as taking a very long time and low reproducibility. In immunological techniques, there are problems such as a high risk of contamination and low sensitivity levels. Molecular biological techniques, on the other hand, have emerged as an alternative to the developing

technology and they have been preferred due to their high sensitivity, high repeatability, and fast results (Tutar et al., 2015; Rathnayaka et al., 2018; Agrimonti et al., 2019).

Kılıç Altun et al. (2017) investigated the presence of *Listeria spp.* by Real-Time PCR in yoghurts sold in Şanlıurfa/Turkey in their study in 2016. As a result of the study performed on 62 yogurt samples, the prevalence of *Listeria spp.* was found to be 3.2%. In another study conducted in Şanlıurfa/Turkey in 2017 Deniz and Kılıç Altun, (2017) tried to detect *Listeria monocytogenes* contamination in regional cheeses produced from raw sheep milk and raw cow milk with Real-Time PCR. As a result of the Listeriolysin gene region scanning in 97 cheese samples, *Listeria monocytogenes* were detected in 3 samples. Liming et al. (2004) conducted a study investigating the presence of *Listeria monocytogenes* in products such as fresh-cut melon and mixed salad using a Real-Time PCR. In the study, the classical colony method, conventional PCR, and Real-Time PCR methods were compared. As a result of the comparison, it was determined that the analysis with the Real-Time PCR method gave results in 26 hours less than the conventional method.

Other analysis

Ferreira et al. (2016) investigated the presence of barley, corn, and rice genes by Real-Time PCR method in their study on soluble coffee products in 2016. While 30 coffee products were collected from different countries and different grains were not found in the products defined as top quality, the grains analyzed were found in lower quality products collected from South America.

Gansbeke et al. (2018) investigated the mixture of apricot kernel paste in almond butter products. Within the scope of the research, apricot kernel paste in different proportions was added to the almond paste and the detection limit study and optimization study were carried out.

Kabacaoğlu and Karakaş Budak, (2019) developed a method for detecting adulteration by Real-Time PCR in the salep product that can be mixed with cornflour or potato starch. They were able to measure up to 2 levels of DNA level with high precision using the primer sets they designed.

Sobrino-Gregorio et al. (2019) conducted a study to determine whether sugar is added from different plant sources in honey with the Real-Time PCR method. In the study, different amounts of rice molasses were added to the natural honey sample and it was aimed to determine the mixing ratios with the developed method.

Mohamad et al. (2018) conducted a study in 2018 on the detection of pig genes in gelatin and gelatin capsules with the Real-Time PCR method. As a result of the study, it has been observed that working with chromosomal DNA in intensely processed products such as gelatin is more efficient than mitochondrial DNA and a method has been developed for the halal food tests of various foods on the market.

Villa et al. (2017) worked on the development of a Real-Time PCR-based method to detect possible adulterations in saffron plant products. As a result of the study, a method has been developed in which the safflower mixture that can be made into saffron products such as stigma, spices, and powders can be determined.

Conclusion

Molecular-based analysis techniques will be used more widely in the future in parallel with biotechnological developments. In the analysis using Real-Time PCR methods, the nucleic acid-containing the targeted gene region must be extracted in sufficient quality and concentration for the analysis to be successful. Correct nucleic acid isolation is the basis of the analysis mentioned in this research. Real-time PCR reactions, starting with the appropriate amount and purity nucleic acid solution and error-free primer-probe sequence, will give results with high repeatability and precision.

In food quality control laboratories, quantitative methods are routinely used in analyzes such as GMO and pathogen microorganism analysis; however, qualitative methods are widely used in analyses such as meat-type determination, milk origin determination, and allergen analysis. On the other hand, an important step can be taken to prevent possible adulteration in food by increasing the frequency of use of biotechnological analysis methods such as Real-Time PCR methods. Although there are studies with quantitation content mentioned in this study, there are no studies on the usability of the methods used in these studies in routine analysis. In such analyzes, it is thought that there is a low level of repeatability in front of the transition to quantitation. On the other hand, it is thought that the efficiency increase studies in nucleic acid extraction will be valuable for the routine quantitation analysis.

Author Contributions

EA: designed, performed, writing; SA: review and editing

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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