부정·불량식품 안전관리분야
전문성 향상을 위한 감시기법 연구

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식품의약품안전처

강태선

목 차

국외훈련개요

1. 훈련국: 캐나다

2. 훈련기관명: 피오에스 바이오 사이언스 (POS BIO-SCIENCE)

3. 훈련분야: 식품안전관리

(훈련과제: 부정·불량식품 안전관리분야 전문성 향상을 위한 감시기법 연구)

4. 훈련기간: 2017.4.21. – 2018.4.20.

훈련기관개요

1. 기관개요

○ 훈련국: 캐나다

○ 훈련기관명: POS BIO-SCIENCES

○ 인터넷 웹주소: www.pos.ca

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- President and Chief Executive Officer of POS BIO SCIENCES
- Vice President, Agriculture, Biotechnology, and Food Division at the Saskatchewan Research
- President and CEO of AgraPoint International

2. 기관소개

○ 설립 목적

▪ POS bio-sciences 는 다양한 생물재료로부터 고부가가치의 식품원료, 의약품원료 생산 공정을 연구·개발하는 글로벌 선도 기업으로 연구개발, 분석업무, 고객맞춤형 공정 개발 등 양질의 서비스를 제공하고자 함

▪ 1973 년도에 공기업으로 설립되어 2012 년에 민영화되어 현재 50 개국 이상에서 5,400 건 이상의 프로젝트를 수행 중에 있음

○ 조직

▪ POS bio-sciences 는 미국(Batavia)과 캐나다(Saskatoon)에 현지공장을 다음과 같은 유닛으로 운영하고 있음

- 공정 개발 분야(Process Development)
- 고객 맞춤형 공정 개발 분야(Custom Processing)
- 분석 서비스 분야(Analytical Services)
- 기술지원 및 기술상담 분야(Project Support)
- POS bio-sciences 캐나다는 11 개의 자체 실험실을 보유하고 있으며, 캐나다 종합대학교, 정부기관 등과의 공동연구 프로그램을 운영하고 있음

3. 주요기능 및 연구분야

○ 농업 및 식용작물 분야에서 분자생물학 및 바이오텍 기술을 접목하여 분자 수준에서의 기능성 원료개발, 의약품 원료개발, 종 판별, 공정개발 등에 관한 연구가 활발히 이루어지고 있음

▪ Agricultural Biotechnology: 바이오텍 기술을 이용한 식용작물의 부가가치 극대화 연구

▪ Food Processing: 식품가공공정 개발을 통한 고부가가치의 식품원료 개발 연구

▪ Nutraceuticals: 생리활성물질, 항산화물질 등 기능성 식품원료 개발 연구

▪ Pharmaceuticals and Medical ingredients: 미국 식품의약품안전청 등의 기준에 적합한 의약품 원료물질 개발 연구

▪ Industrial Bio-Products: 산업체 폐기물 또는 부산물을 바이오 연료 등의 고부가가치 물질 전환 연구

▪ Cosmetics and Toiletries: 인공합성 또는 석유 기반의 기능성 물질을 대체할 수 있는 천연 항산화물질, 향료, 왁스, 단백질 등 생리활성물질의 추출 및 생합성 연구

Chapter 1[i]

Basic principles for developing real-time PCR methods used in food analysis

[i] This chapter is submitted to Trends in Food Science and Technology. Tae Sun Kang. 2018. Basic principles for developing real-time PCR methods used in food analysis: a review.

1.1 Abstract

Background: The increased interest in global food fraud has led to the development of a number of advanced methods, among which real-time polymerase chain reaction (PCR) currently plays an integral role in food authentication. However, the lack of standard parameters for the development and validation of real-time PCR methods hampers their utilization across different laboratories and conditions, leading to inconsistent results.

Scope and Approach: This review summarizes and assesses different methods presented throughout a large number of scientific papers, including DNA extraction, primer design, and quantification (or qualification) as well as parameters for the development and validation of real-time PCR methods in food analysis.

Key Findings and Conclusions: Inhibitors in DNA extracts can cause decreased PCR sensitivity and false negative results; thus, the use of PCR inhibition and amplification controls (e.g., the 18S ribosomal RNA gene) is essential for obtaining accurate real-time PCR results. In quantitative real-time PCR methods, the results obtained using species-specific systems need to be normalized by using reference systems for the improvement of their accuracy. Therefore, this review will provide researchers with a beneficial guide for the development of real-time PCR methods in a harmonious manner and contribute to an enhanced applicability of the methods developed.

Keywords : Real-time PCR; Food analysis; Food fraud; Species identification; Validation; Quantification; Method acceptance parameters; PCR inhibition; PCR amplification control

1.2 Introduction

Food fraud, which constitutes illegal deception for economic gain using food, has become a global concern that can deeply impact governments, industry, and consumers. For example, the incidents involving melamine in 2008 and horse meat in 2013 clearly demonstrate how adulteration negatively influences global food safety and economy (Moyer, DeVries, & Spink, 2017). In addition, the development of a processed-food market, owing to improvements in food-processing technologies, has led to an increase in economically motivated adulteration (EMA) of food, such as the substitution of a valuable authentic constituent with a less expensive ingredient, or the false declaration of either raw material origin or the production process used to manufacture an ingredient. Such fraudulent practices have raised concerns for health safety as well as religious beliefs.

Traditionally, identification methods rely heavily on morphological characteristics, such as shape, color, odor, and texture of food or food ingredients; however, these approaches are rarely useful for processed products because of the destruction of original observable characteristics. Therefore, development of advanced detection methods constitutes an important first line of defense for both detecting and deterring food fraud. A study analyzing food fraud from 1908 to 2010 found that 16 technologies were commonly used for detecting food adulteration (Moore, Spink, & Lipp, 2012). Among these, the top 7 comprised high-performance liquid chromatography (HPLC), infrared spectroscopy (IR), gas chromatography (GC), isotope ratio mass spectrometry (IRMS), hyphenated mass spectroscopy (MS), near infrared spectroscopy (NIR), and polymerase chain reaction (PCR). However, numerous secondary metabolites present in processed products render the chromatographic and spectral fingerprints difficult to analyze various types of food.

As a consequence, DNA-based techniques have recently been highlighted for the authentication of processed food, owing to their sensitivity and accuracy regardless of growing condition, harvesting period, and manufacturing process. Various PCR-based techniques have been widely used for the identification of commercially important species, such as DNA barcoding (Hellberg & Morrissey, 2011; Rasmussen & Morrissey, 2008), forensically informative nucleotide sequencing (FINS) (Bartlett & Davidson, 1992), microsatellite analysis (Kang, Park, & Jo, 2012; Kempter, Kielpinski, Panicz, Pruffer, & Keszka, 2017), PCR-restriction fragment length polymorphism (PCR-RFLP) analysis (Cho, Kim, Kim, Kang, Dong, An, et al., 2014; Wilwet, Jeyasekaran, Shakila, Sivaraman, & Padmavathy, 2017), and species-specific PCR (Lee, Kim, Jo, Jung, Kwon, & Kang, 2016; Wen, Hu, Zhang, & Fan, 2012). Real-time PCR, a more recently developed technique, can detect multiple species from a mixture and quantify the amount of PCR products formed during the amplification process (Arya, Shergill, Williamson, Gommersall, Arya, & Patel, 2005). Moreover, its reliability, sensitivity, specificity, and rapidity are suitable for the development of advanced detection methods to regulate EMA foods. Real-time PCR systems have been previously used for the authentication of various foods including meat products, seafood products, dairy products, spices, and dietary supplements (Amaral, Santos, Oliveira, & Mafra, 2017; Di Pinto, Terio, Marchetti, Bottaro, Mottola, Bozzo, et al., 2000; J. H. Kim, Moon, Kang, Kwon, & Jang, 2017; Taboada, Sanchez, & Sotelo, 2017). However, despite the increased need for real-time PCR in food analysis, basic principles for method development and validation have yet to be comprehensively suggested or fully evaluated.

The aim of this review is to provide essential guidelines for the development of real-time PCR methods that can be utilized to assess food authenticity or detect food fraud. Toward this end, this study extracted the different techniques and criteria from a large number of scientific papers and evaluated their strengths, limitations, and applications. In this

review, analytical targets for real-time PCR methods were limited to food and/or food ingredients, whereas genetically modified organisms (GMO), food-borne bacteria, and other pathogens were excluded, because the associated criteria for real-time PCR analysis have already been well documented (Broeders, Huber, Grohmann, Berben, Taverniers, Mazzara, et al., 2014; ENGL, 2015; Kralik & Ricchi, 2017). In addition, almost all of the real-time PCR methods for food analysis published in scientific articles were evaluated by in-house validation using acceptance parameters only; therefore, in this review, the performance parameters verified by collaborative studies were discounted. A general review on method performance parameters can be found in two previous articles related to GMO detection by Broeders et al. (2014) and ENGL (2015). In the following sections, different methods of DNA extraction and quantification, controls for PCR inhibition and amplification, and selection of primers and proves are discussed. Various parameters, such as specificity, sensitivity, and efficiency that are necessary for the development of real-time PCR methods are reviewed. Finally, different methods for quantification and validation (accuracy, precision, and robustness) are compared and assessed.

1.3. DNA extraction, concentration, and purity

1.3.1 DNA extraction methods

Isolation of genomic DNA from food or food ingredients constitutes the first key step for successful real-time PCR results. In previous studies, different DNA extraction methods, including commercial kits and in-house methods, were compared for the analysis of meat, fish, and plant products. To ascertain the best product analysis, six in-house methods including Tris-ethylenediaminetetraacetic acid (EDTA), modified cetyltrimethylammonium bromide (CTAB), and alkaline, urea, salt, guanidinium isothiocyanate (GuSCN) methods, as well as four commercial kit methods including Wizard Genomic DNA Purification Kit (Promega, USA), DNeasy Blood & Tissue kit (Qiagen, Germany), Quick-gDNA MiniPrep Kit (Zymo Research, USA), and Genespin DNA Extraction and Purification Kit (Eurofins, Germany), were compared (Yalcinkaya, Yumbul, Mozioglu, & Akgoz, 2017). It was found that the salt method was the most effective in terms of the quantity, purity, and integrity of the DNA extracts, yielding results comparable to those obtained from CTAB and Qiagen methods. In addition, a study conducted by Cawthorn et al. (2011) compared five different methods including modified urea-SDS-proteinase K, modified phenol-chloroform, salt extraction, SureFood PREP Allergen Kit (r-Biopharm, Germany), and Wizard Genomic DNA Purification Kit, for DNA extraction from fish products, and concluded that the SureFood PREP Allergen Kit was the most suitable method. Recently, a fast DNA extraction method without any purification step was developed for seafood species identification (Tagliavia, Nicosia, Salamone, Biondo, Bennici, Mazzola, et al., 2016). All components commonly included in most PCR buffers were used in the lysis butter; thus, variable amounts of crude lysate could be directly used as a template DNA without any inhibitory effects on PCR analysis. For DNA extraction from crops, such as canola, flax, and soybean, seven

commercial kits including Fast ID Genomic DNA Extraction Kit (Genetic ID, USA), FastDNA Spin Kit (MP Biomedicals, USA), Plant DNAzol Reagent (Life Technologies, USA), GM Quicker 2 (Nippon Gene Co., Japan), OminiPrep for Plant (G-Biosciences, USA), NucleoSpin Food (Macherey-Nagel, D-Mark Biosciences, Canada), and DNeasy Mericon Food (Qiagen) were compared with the traditional CTAB method (Demeke, Malabanan, Holigroski, & Eng, 2017). This found that the Fast ID method provided reliable PCR results regardless of species or analysis method used (digital or real-time PCR). Overall, the use of commercial kits is generally preferred for real-time PCR assays owing to their effectiveness and convenience; however, hybrid methods combining the modified CTAB or GuSCN method with commercial clean-up kits have also been widely used for food analysis. These hybrid methods can efficiently remove PCR inhibitors (e.g., polysaccharides and polyphenols) present in processed products and trap short and degraded DNA on the resin membrane. Thus, they can afford higher purity and yield of DNA suitable for real-time PCR assays (Ferreira, Farah, Oliveira, Lima, Vitorio, & Oliveira, 2016; Laube, Zagon, Spiegelberg, Butschke, Kroh, & Broll, 2007; Mujico, Lombardía, Mena, Méndez, & Albar, 2011; Sanchez, Quinteiro, Rey-Mendez, Perez-Martin, & Sotelo, 2009; Taboada, Sanchez, & Sotelo, 2017).

1.3.2 DNA quantification methods

The quantity and purity of DNA extracts are critical factors dominating the results of real-time PCR. DNA quantification is typically measured by either spectrophotometric or fluorometric methods, with the former representing the most commonly used technique. The absorbance of DNA extracts is measured at 260 nm (A_{260}) and the concentration and yield are calculated using the following equations, assuming that 1 absorbance unit corresponds to 50 µg/mL double-stranded DNA (dsDNA) (Sambrook & Russell, 2001) : Concentration (ng/µL) = A_{260} × 50 × dilution factor and Yield (µg) = concentration × the total volume of DNA

extract. However, this spectrophotometric method is sensitive to single stranded DNA, RNA, proteins, and organic contaminants (such as chloroform and phenol) commonly found in isolated DNA, which can lead to the marked overestimation of DNA quantity according to the different chemical characteristics of the target materials (J. Costa, Amaral, Grazina, Oliveira, & Mafra, 2017; Scarafoni, Ronchi, & Duranti, 2009). In contrast, the fluorometric method employs fluorescent dyes specific to dsDNA, the fluorescence of which is measured and used to calculate the amount of dsDNA present in the DNA extract; thus, this method can be used for real-time PCR methods requiring high sensitivity for processed food products. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of extracted DNA. Generally, a ratio over 1.8 is accepted as pure DNA that can be used for real-time PCR analysis (Demeke, Malabanan, Holigroski, & Eng, 2017; Taboada, Sanchez, & Sotelo, 2017; Yalcinkaya, Yumbul, Mozioglu, & Akgoz, 2017). Lower ratios indicate the presence of protein, phenol, or other contaminants that absorb strongly near 280 nm. The ratio of absorbance at 260 nm and 230 nm can be used as a secondary measure of DNA purity. Acceptable values for real-time PCR analysis are commonly in the range of 2.0–2.2, indicating the absence of contaminants (e.g., carbohydrates, phenol, and EDTA) that absorb near 230 nm (Demeke, Malabanan, Holigroski, & Eng, 2017; Yalcinkaya, Yumbul, Mozioglu, & Akgoz, 2017). It should be noted that these values are strongly dependent on the matrices used; thus, lower values can be empirically acceptable for the analysis of highly processed foods.

1.4 PCR inhibition and amplification controls

PCR inhibitors comprise a heterogeneous group of organic (such as detergents, polyphenols, polysaccharides, and proteins) and inorganic (such as calcium ions and salts) substances. They can originate from a variety of food ingredients or be introduced during food processing or DNA isolation. Generally, the PCR reaction is sensitive to inhibitory substances, which can lead to a decrease in PCR sensitivity or false negative results. These inhibitors can additionally contribute to interference with fluorescent probes or increased background fluorescence in real-time PCR assays (Schrader, Schielke, Ellerbroek, & Johne, 2012). Therefore, for successful real-time PCR results, it is important to assess the potential inhibition of PCR via different types of controls that can be classified by when and how they are implemented, as follows: (1) competitive and non-competitive controls are amplified by the same and different primers as the target, respectively, (2) internal and external controls are analyzed in the same and different tubes as the target, respectively, and (3) process and amplification controls wherein defined amounts of DNA are added at the beginning of sample preparation steps (e.g., before DNA extraction) and at the PCR reaction step, respectively (Schrader, Schielke, Ellerbroek, & Johne, 2012).

Various types of internal controls have been widely used to assess inhibition and false negative results that occur during real-time PCR analysis. For example, a competitive internal amplification control constituting a recombinant plasmid including a DNA fragment with the same primer binding sites but different size and sequence as the target, was used to develop real-time PCR methods for the detection of peanut allergens and murine meat in food (Fang & Zhang, 2016; Zhang, Cai, Guan, & Chen, 2015). A non-competitive internal amplification control; i.e., a recombinant plasmid with different primer binding sites and sequence as the target, was used to develop real-time PCR for the simultaneous detection of seven animal

species (Laube, Zagon, Spiegelberg, Butschke, Kroh, & Broll, 2007). Moreover, a study conducted by Holzhauser et al. (2014) used a competitive internal process control, involving a DNA fragment with conserved primer binding sites but different probe binding site and amplicon size, for the quantitative detection of peanut in food using real-time PCR. In this study, a defined amount of the DNA competitor was spiked with food samples prior to DNA extraction, and the effects of DNA extraction and amplification on the PCR efficiency were assessed based on the quantification cycle (Cq) value observed for the competitor.

DNA degradation caused during manufacturing processes (e.g., high temperature and pressure) usually results in a decrease in amplifiable DNA; thus, the degree of DNA degradation should be considered when developing reliable real-time PCR methods for processed food products. As reference genes originate directly from the samples, they can act as internal or external non-competitive process controls. Therefore, the use of reference genes represents the most preferred control system to assess the presence of both amplifiable DNA and PCR inhibitors. Various reference genes (e.g., 18S rRNA and myostatin) have been reported in previous studies, among which the 18S ribosomal RNA (18S rRNA) gene was most widely used in reference systems for qualitative and quantitative real-time PCR methods (Table 1.1). Various universal primer and probe sets have been developed to amplify conserved sequences of the 18S rRNA gene from eukaryotic organisms, producing short amplicon sizes from 77 to 141 bp (Table 1.1). This reference gene has been successfully used as an external control that assesses amplification, inhibition, and false negative results of real-time PCR methods for crustacean, poultry, and plant species detection (J. H. Kim, Moon, Kang, Kwon, & Jang, 2017; N. Pegels, Gonzalez, Garcia, & Martin, 2014; Zagon, Schmidt, Schmidt, Broll, Lampen, Seidler, et al., 2017; Zhang, Cai, Guan, & Chen, 2015). In addition, this system has been used as an internal control to develop a multiplex real-time PCR assay for the simultaneous detection of cattle, buffalo, and porcine DNA (Hossain, Ali, Sultana,

Asing, Bonny, Kader, et al., 2017). For quantitative real-time PCR, reference systems can be used for amplification and inhibition controls as well as for normalization of the results obtained using the species-specific systems (discussed further in Section 1.7). In particular, Laube, Zagon, Spiegelberg, et al. (2007) reported a reference system based on the single-copy myostatin gene, which is highly similar in mammalian and poultry species, as an amplification control and for quantitative normalization.

Additionally, the presence of PCR inhibitors can be detected by spiking samples with positive controls or using dilutions of the extracted DNA. In real-time PCR assays for the detection of celery and/or mustard species in processed products, food samples that had tested as negative were subjected to inhibition control reactions in which DNA extracted from the negatively-tested food samples was spiked with positive control DNA (e.g., reference celery and or mustard), and their Cq values were compared with those of positive controls (Magdalena Fuchs, Cichna-Markl, & Hochegger, 2012; M. Fuchs, Cichna-Markl, & Hochegger, 2013; Palle-Reisch, Hochegger, & Cichna-Markl, 2015). In comparison, the dilution method, which automatically dilutes PCR inhibitors, is more widely used because inhibitory substances in DNA may not act equally in different RCR reactions (Bustin, Benes, Garson, Hellemans, Huggett, Kubista, et al., 2009; Schrader, Schielke, Ellerbroek, & Johne, 2012). In particular, the European Union Reference Laboratory for Animal Proteins (EURL-AP) real-time PCR protocol for horse meat detection recommended that the ΔCq value between two ten-fold dilutions never exceed 0.8, which was considered as acceptable (EURL-AP, 2013). In acceptance criterion for GMO detection, the average ΔCq value (measured Cq − extrapolated Cq) of the first diluted sample of the inhibition test should be less than 0.5 (ENGL, 2015) (discussed further in Section 1.6.2.2).

Table 1.1 Summary of reference systems used in real-time PCR methods for food analysis.

	Target genes	Primer/Probe sequence (5' to 3')	Size (bp)	Purpose	Target species	References
1	18S rRNA	F: CTGCCCTATCAACTTTCGATGGTA R: TTGGATGTGGTAGCCGTTTCTCA P: ACGGGTAACGGGGAATCAGGGTTCGATT	113	Amplification, inhibition, and normalization controls	Eukaryotic organisms	Amaral et al. (2017) Costa et al. (2013) Costa et al. (2017) Meira et al. (2017)
2	18S rRNA	F: TCGATGGTAGGATAGTGGCCTACT R: TGCTGCCTTCCTTGGATGTGGTA	109	Amplification, inhibition, and normalization controls	Eukaryotic organisms	Villa et al. (2017)
3	18S rRNA	F: GGTAGTGACGAAAAATAACAATACAGGAC R: ATACGCTATTGGAGCTGGAATTACC P: AAGTGGACTCATTCCAATTACAGGGCCT	141	Amplification, inhibition, and normalization controls	Eukaryotic organisms	Hossain et al. (2017) Kim et al. (2016) Rojas et al. (2010)
4	18S rRNA	F: TCTGCCCTATCAACTTTCGATGG R: TAATTTGCGCGCCTGCTG	140	Amplification, inhibition, and normalization controls	Eukaryotic organisms	Fajardo et al. (2008)
5	18S rRNA	F: AGCCTGCGGCTTAATTTGAC R: CAACTAAGAACGGCCATGCA P: AGGATTGACAGATTGAG	120	Amplification, inhibition, and normalization controls	Eukaryotic organisms	López-Andreo et al. (2005)
6	18S rRNA	F: TGGTGCCAGCAGCCGC R: TCCAACTACGAGCTTTTTAACTGCA P: CGCTATTGGAGCTGGAATTACC	77	Amplification and inhibition controls	Eukaryotic organisms	de la Cruz et al. (2013) López-Calleja et al. (2013) López-Calleja et al. (2015) Pegels et al. (2011) Pegels et al. (2014)
7	18S rRNA	F: GTAATTTGCGCGCCTGCT R: GTTCGATTCCGGAGAGGGA P: CCTTCCTTGGATGTGGTAGCCGTTTCTC	N.I.	Amplification and inhibition controls	Eukaryotic organisms	Zagon et al. (2017)

8	18S rRNA	F: TCTGCCCTATCAACTTTCGATGGTA R: AATTTGCGCGCCTGCTGCCTTCCTT	137	Amplification and inhibition controls	Eukaryotic organisms	Kim et al. (2017) Zhang et al. (2015)
9	18S rRNA	F: GGCTCATTAAATCAGTTATG R: CCGAGTTATCTAGAGTCA P: CCGTACTTGGATAACTGTGGCAATTC	N.I.	Amplification and inhibition controls	Fish	Herrero et al. (2014)
10	TrnL	F: CGAAATCGGTAGACGCTACG R: GGGGATAGAGGGATTGAAC	N.I.	Amplification and inhibition controls	Plant	Scarafoni et al. (2009)
11	Myostatin	F: TTGTGCAR$_{(A\ and\ G)}$ATCCTGAGACTCAT R: ATACCAGTGCCTGGGTTCAT P: CCCATGAAAGACGGTACAAGR$_{(A\ and\ G)}$TATACTG	97	Amplification, inhibition, and normalization controls	Animal and poultry	Druml, Grandits, et al. (2015) Druml, Hochegger, et al. (2015) Druml, Mayer, et al. (2015) Iwobi et al. (2015) Laube, Zagon, Spiegelberg, et al. (2007)
12	Myostatin	F: TTGTGCAR$_{(A\ and\ G)}$ATCCTGAGACTCAT R: TTCAR$_{(A\ and\ G)}$AGATCGR$_{(A\ and\ G)}$ATTCCAGTATA P: CCCATGAAAGACGGTACAAG	70	Amplification, inhibition, and normalization controls	Animal and poultry	Druml et al., (2016)

N.I., not indicated; F, forward primer; R, reverse primer; P, probe.

1.5 Selection of target genes and design of primers and probes

As discussed in Sections 1.3 and 1.4, DNA degradation into small fragments constitutes a key factor governing successful real-time PCR results. In particular, the degree of DNA fragmentation in processed wheat, maize, and soy bean products has been quantified using a DNA fragmentation index (DFI: 0 to 1), with the higher indices (i.e., smaller fragments) being found following isolation procedures utilizing greater heating time and temperature (Mano, Nishitsuji, Kikuchi, Fukudome, Hayashida, Kawakami, et al., 2017). As shown in Table 1.2, various genetic markers, such as 12S ribosomal RNA (12S rRNA), 16S ribosomal RNA (16S rRNA), cytochrome b (cytb), and cytochrome oxidase I (COI) genes as well as internal transcribed spacers (ITSs) and displacement loop (D-loop) regions, have been widely used for mammalian, poultry, fish, and plant species identification as they are present in high copy number, compared with that of nuclear DNA, and are highly conserved, enabling the design of specific and sensitive primer/probe sets (Bartlett & Davidson, 1991). The use of these multi-copy genes allows real-time PCR methods with a lower limit of detection (LOD) as compared with single-copy genes (Table 1.2). For example, for the reliable quantitative analysis of meat products, species-specific primers and probes were designed based on single-copy DNA targets such as the promoter region of the lactoferrin gene for roe deer (Druml, Mayer, Cichna-Markl, & Hochegger, 2015), epidermal growth factor pseudogene for fallow, red, and sika deer (Druml, Grandits, Mayer, Hochegger, & Cichna-Markl, 2015), non-coding region of the cyclic GMP phosphodiesterase gene for cattle, lamb, and goat, non-coding region of the ryanodine receptor gene for pork, and interleukin-2 precursor gene for chicken, turkey, and duck (Laube, Zagon, Spiegelberg, Butschke, Kroh, & Broll, 2007). For the detection of potential food allergens, the genes coding for allergenic proteins have also been used as target sequences, including the 2S albumin gene for brazil nut

(de la Cruz, López-Calleja, Alcocer, González, Martín, & García, 2013), Cor a 1, 8, 9, 11, 13, and 14 genes for hazelnut (D'Andrea, Coïsson, Locatelli, Garino, Cereti, & Arlorio, 2011; Iniesto, Jimenez, Prieto, Cabanillas, Burbano, Pedrosa, et al., 2013), Ara h1 gene for peanut (Zhang, Cai, Guan, & Chen, 2015), Pru 1 gene for almond (Pafundo, Gullì, & Marmiroli, 2009), and Jug r 3 gene for walnut (Joana Costa, Oliveira, & Mafra, 2013).

Amplicon size constitutes another important factor along with DNA amount for improving assay performance, with amplification of the small fragments of multi-copy DNA targets usually recommended for the analysis of degraded DNA from processed food samples. Prior examination of the effect of amplicon size on real-time PCR results showed that the sensitivity of primers targeting a 155-bp fragment was 1000 times higher than that of primers targeting a 382-bp fragment in the detection of safflower DNA (Villa, Costa, Oliveira, & Mafra, 2017). Moreover, in raw wheat samples, similar Cq values were observed for all amplicon sizes (100, 200, 400, and 800 bp), whereas Cq values were progressively increased in accordance with amplicon size in heat-treated wheat samples (Mano, et al., 2017). Therefore, to increase the sensitivity (LOD) of real-time PCR assays, in the majority of prior studies primers have been generally designed to amplify fragments less than 150 bp (Table 1.2).

Table 1.2 Summary of target genes and their sensitivity and amplicon size.

Target		Amplicon size (bp)	Sensitivity		Reference
Gene	**Species**		**LOD**	**LOQ**	
Mitochondrial 12S rRNA	Quail	129	84 fg diluted DNA (raw) 1.3 pg diluted DNA (heated)	-	Rojas et al. (2010)
	Pheasant	113	16.2 fg diluted DNA (raw) 64.5 fg diluted DNA (heated)	-	
	Partridge	141	20.8 fg diluted DNA (raw) 408 fg diluted DNA (heated)	-	
	Guinea fowl	130	4.2 fg diluted DNA (raw) 36.8 fg diluted DNA (heated)	-	
	Pigeon	133	13.6 fg diluted DNA (raw) 12.1 fg diluted DNA (heated)	-	
	Eurasian woodcock	138	67 fg diluted DNA (raw) 538 fg diluted DNA (heated)	-	
	Song thrush	110	3.3 fg diluted DNA (raw) 32 fg diluted DNA (heated)	-	
	Red deer	134	-	0.001% w/w (raw) 0.8% w/w (heated)	Fajardo et al. (2008)
	Fallow deer	169	-	0.00006% w/w (raw) 0.0014% w/w (heated)	
	Roe deer	120	-	0.00004% w/w (raw) 0.00033% w/w (heated)	
Mitochondrial 16S rRNA	Shrimps	80	0.25 pg diluted DNA (raw)	-	Zagon et al. (2015)
	Lobster	85	0.04 pg diluted DNA (raw)	-	
	North sea shrimp	80	1.1 pg diluted DNA (raw)	-	
	Chinese mitten crab	85	0.25 pg diluted DNA (raw)	-	
	River prawns	78	0.25 pg diluted DNA (raw)	-	

	Northern prawn	85	2.5 pg diluted DNA (raw)	-	
Mitochondrial D-loop	Pork	83	0.01 pg diluted DNA (raw) 0.1 pg diluted DNA (heated)	1 pg diluted DNA or 0.1% w/w (heated)	Kim et al. (2016)
	Cattle	76	-	0.1% w/w (heated)	Pegels et al. (2011)
	Sheep	77	-	0.1% w/w (heated)	
Mitochondrial cytochrome b	Horse	141	0.1 pg diluted DNA or 0.0001% w/w (heated)	0.0001% w/w (heated)	Meira et al. (2017)
	Pork	107	0.01 pg diluted DNA or 0.0001% w/w (heated)	0.01% w/w (heated)	Amaral et al. (2017)
	Buffalo	90	1 pg diluted DNA (raw)	-	Hossain et al. (2017)
	Pork	146	1 pg diluted DNA (raw)	-	
	Cattle	92	0.03 pg diluted DNA (raw)	-	López-Andreo et al. (2005)
	Pork	100	0.07 pg diluted DNA (raw)	-	
	Sheep	119	0.07 pg diluted DNA (raw)	-	
	Chicken	117	0.006 pg diluted DNA (raw)	-	
	Turkey	146	0.80 pg diluted DNA (raw)	-	
	Ostrich	120	0.03 pg diluted DNA (raw)	-	
	Pigeon	106	0.01% w/w (heated)	-	Kim et al. (2018)
	Chicken	133	0.01% w/w (heated)	-	
	Ling	81	0.1 pg diluted DNA (raw)	-	Taboada et al. (2017)
Mitochondrial cytochrome oxidase I	Atlantic cod	140	20 pg diluted DNA (raw)	-	Herrero et al. (2010)
	Atlantic mackerel	60	0.1 pg diluted DNA (raw)	-	Velasco et al. (2013)
Internal transcribed	Hazelnut	70	0.1 ppm (heated)	-	López-Calleja et al. (2013)

spacer	Walnut	70	0.1 ppm (heated)	-	López-Calleja et al. (2015)
	Pecan	69	0.1 ppm (heated)	-	
	Safflower	155	2 pg diluted DNA or 0.1% w/w (raw)	0.1% w/w (raw)	Villa et al. (2017)
Lactoferrin	Roe deer	62	0.03% w/w (raw)	0.5% w/w (raw)	Druml, Mayer, et al. (2015)
Epidermal growth factor pseudogene	Fallow, red, and sika deer	68	0.1% w/w (raw)	0.1% (sika) - 0.5% w/w (raw)	Druml, Grandits, et al. (2015)
Cyclic GMP phosphodiesterase	Cattle	102	10 genome copies diluted DNA (raw)	-	Laube, Zagon, Spiegelberg, et al. (2007)
	Lamb	97	10 genome copies diluted DNA (raw)	-	
	Goat	96	10 genome copies diluted DNA (raw)	-	
Ryanodine receptor	Pork	108	10 genome copies diluted DNA (raw)	-	
Interleukin 2 precursor	Chicken	95	10 genome copies diluted DNA (raw)	-	
	Turkey	86	10 genome copies diluted DNA (raw)	-	
	Duck	212	10 genome copies diluted DNA (raw)	-	
2S albumin	Brazil nut	131	2.5 ppm (heated)	-	de la Cruz et al. (2013)
Cor a 1	Hazelnut	105	0.001% w/w (raw)	-	D'Andrea et al. (2011)
Cor a 8		78	0.001% w/w (raw)	-	
Cor a 14		116	0.001% w/w (raw)	-	
Cor a 9	Hazelnut	101	0.0001% w/w (raw)	-	Iniesto et al. (2013)
Cor a 11		101	0.0001% w/w (raw)	-	
Cor a 13		101	0.0001% w/w (raw)	-	
Ara h 1	Peanut	179	0.005% w/w (raw)	-	Zhang et al. (2015)
Pru 1	Almond	76	1 genome copy diluted DNA (raw)	-	Pafundo et al. (2009)
Jug r 3	Walnut	99	1 pg diluted DNA or 0.001% w/w (heated)	0.001% w/w (heated)	Costa et al. (2013)

1.6 Parameters for qualitative and quantitative real-time PCR

When developing real-time PCR (single, multiplex, qualitative, and/or quantitative) methods for food analysis, the analytical scope and aim should be considered, by which adequate parameters and strategies are carefully adapted (Table 1.3). For the development of a multiplex real-time PCR method, it is further necessary to show evidence demonstrating that the following parameters are not impaired in comparison with those of each single method.

1.6.1 Analytical specificity

The specificity of primer and/or probe sets used for real-time PCR can be confirmed by *in silico* and experimental (*in situ*) analyses. First, the theoretical specificity is assessed by comparing the amplicon sequences with those of unintended species available through GenBank using the Basic Local Alignment Search Tool (BLAST) and/or primer-BLAST tool (Ye, Coulouris, Zaretskaya, Cutcutache, Rozen, & Madden, 2012), for suggesting possible cross-reaction information. Second, the cross-reactivity of chosen primer/probe sets may be experimentally tested against genetically related species or other species that can be commonly employed as food ingredients during manufacturing processes. Positive results from this analysis suggest unexpected cross-reactivity or cut-off Cq values that can be used to differentiate between target and non-target species (Druml, Grandits, Mayer, Hochegger, & Cichna-Markl, 2015; Druml, Mayer, Cichna-Markl, & Hochegger, 2015; Espineira & Vieites, 2012; Iniesto, et al., 2013; López-Andreo, Lugo, Garrido-Pertierra, Prieto, & Puyet, 2005; Taboada, Sanchez, & Sotelo, 2017). For plant species, different cultivars need to be experimentally tested for specificity, because intra-specific polymorphisms or site-specific point mutations may constitute a potential source of false negative results (D'Andrea, Coïsson,

Locatelli, Garino, Cereti, & Arlorio, 2011; M. Fuchs, Cichna-Markl, & Hochegger, 2013). In the case of DNA intercalating dye-based real-time PCR methods (e.g., SYBR Green I), the melting temperature (Tm) should be suggested as an additional parameter for PCR specificity, because this chemistry can produce false signals from non-specific amplicons and primer-dimers formed during PCR reaction (Alonso-Rebollo, Ramos-Gomez, Busto, & Ortega, 2017; Amaral, Santos, Oliveira, & Mafra, 2017; D'Andrea, Coïsson, Locatelli, Garino, Cereti, & Arlorio, 2011; Fajardo, Gonzalez, Martin, Rojas, Hernandez, Garcia, et al., 2008; Iniesto, et al., 2013; Meira, Costa, Villa, Ramos, Oliveira, & Mafra, 2017; Mujico, Lombardía, Mena, Méndez, & Albar, 2011; Ramos-Gomez, Busto, Albillos, & Ortega, 2016; Scarafoni, Ronchi, & Duranti, 2009; Villa, Costa, Oliveira, & Mafra, 2017). Additionally, the specificity of real-time PCR methods is further verified by examining the expected size and sequence of PCR products by gel electrophoresis and sequencing, respectively.

Table 1.3 Parameters for development of qualitative and quantitative real-time PCR in food analysis (acceptable parameters in parentheses).

Step	Parameter	Real-time PCR methods	
		Qualitative analysis	**Quantitative analysis**
DNA integrity	Concentration, yield, and purity	+	+
Amplification	Amplifiability	+	+
	Inhibition	+	+
In-house validation	Specificity	+ (*in silico* and *in situ* specificity)	+ (*in silico* and *in situ* specificity)
	Linear dynamic range[a]	+ (5 to 6 $\log_{10}$ concentrations)	+ (5 to 6 $\log_{10}$ concentrations)
	Amplification efficiency	+（90% to 110%）	+（90% to 110%）
	Linearity	+（$R^2 \geq 0.98$）	+（$R^2 \geq 0.98$）
	Limit of detection	+	+
	Limit of quantification	−	+
	Trueness	−	+（Bias ≤ ±25~30%）
	Precision (Repeatability)	−	+（RSDr ≤ 25%）
	Robustness[b]	+（Trueness and Repeatability ≤ 30%）	+（Detection of the target）
Quantification (result expression)	Normalization by reference systems	− (Presence or absence by the cut-off Cq values)	+ (DNA concentration or relative amount)

a. b. Values recommended by the MIQE (Bustin et al. 2009) and GMO (ENGL, 2015) guidelines, respectively.
+, parameter to be evaluated; −, parameter not to be evaluated

1.6.2 Amplification efficiency

1.6.2.1 Standard curves

In real-time PCR assays, standard curves can act as a simple, rapid, and reproducible indicator for evaluating the amplification efficiency and analytical sensitivity (Bustin, et al., 2009). Standard curves are usually plotted using the Cq values and the logarithmic quantities of reference DNA (copy numbers or ng and % concentrations) and are expressed as the following regression equation:

$$y = ax + b$$

where x and y are DNA quantities and Cq values, respectively, and a and b represent slope and intercept (the Cq value at which single-copy DNA could be theoretically detected), respectively. Therefore, linear dynamic range constitutes an important factor for the reliability of standard curves. In previous real-time PCR studies, the dynamic range was generally determined using ten-fold serially diluted DNA to cover four to eight orders of magnitude (Table 1.4). A coefficient of determination (R^2 value) higher than 0.98 was considered as acceptable linearity for the standard curve. The minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines recommended that the dynamic range should cover at least 3 orders of magnitude and ideally should extend to 5 to 6 $\log_{10}$ concentrations (Bustin, et al., 2009). Moreover, in qualitative and quantitative GMO detection, the R value should be over 0.98 (Broeders, et al., 2014; ENGL, 2015).

Table 1.4 Summary of in-house validation parameters used for the development of real-time PCR methods in food analysis.

	Target species	Standard curve				Acceptable validation parameter				Method	Reference
		Dynamic range	Replicate	Linearity (R^2)	Efficiency (%)	Reference material	Trueness[a] (Bias, %)	Repeatability[a] (RSDr, %)	Robustness		
1	Horse	10 ng - 0.1 pg, 6 points	12	0.999	99.6 - 101.8	Model mixtures (0.25, 2.5, 4, 8, 15%; w/w)	≤ ±25% (−32 - +5.5)	≤ 25% (4.4 - 15.5)	N.T.	EvaGreen Single	Meira et al. (2017)
2	Roe deer	387 ng - 24 pg, 8 points	10	0.999	93.9	Model mixtures (2, 10, 25, 38, 50%; w/w)	≤ ±30% (+1.0 - +40.9)	≤ 25% (7.4 - 21.6)	Two Real-time PCR instruments Two Master mixes'	TaqMan Single	Druml, Mayer, et al. (2015)
3	Pork	10 ng - 0.01 pg, 7 points	12	0.996	105.7	Model mixtures (0.25, 2.5, 4, 6%; w/w)	≤ ±25% (−19 - +20.6)	≤ 25% (1.6 - 22.4)	N.T.	EvaGreen Single	Amaral et al. (2017)
4	Safflower	20 ng - 2 pg, 5 points	8	0.999	105.7	Model mixtures (4, 8%; w/w)	≤ ±25% (+16.2 - +16.4)	≤ 25% (4.0 - 6.0)	N.T.	EvaGreen Single	Villa et al. (2017)
5	Cattle, Pork	156250 - 50 genome copy equivalents, 6 points	N.I.	0.999 (cattle) 0.996 (pork)	101.1 (cattle) 91.6 (pork)	Model mixtures (5, 20, 30, 50, 70, 80, 95%; w/w)	≤ ±25% (+1.4 - +11.3)	≤ 25% (0.28 - 11.56)	Three Real-time PCR instruments Two DNA extraction methods	TaqMan Triplex	Iwobi et al. (2015)

6	Soybean	10 ng - 2.44 pg, 7 points	8	0.981 - 0.990	92.2 - 102.7	Model mixtures (0.75, 2.5, 4, 6, 7.5%; w/w)	≤ ±25% (−15.8 - +12.8)	≤ 25% (1.85 - 13.4)	N.T.	TaqMan Single	Costa et al. (2017)
7	Fish	100 ng - 0.01 pg, 8 points	16	0.999	107.4	Model mixtures (0.25, 2.5, 4, 8%; w/w)	≤ ±25% (−5.02 - +19.0)	≤ 25% (7.52 - 15.2)	N.T.	TaqMan Single	Fernandes et al. (2017)
8	Cattle, Buffalo, Pork	10 ng - 1 pg, 4 (cattle) - 5 points	3	0.985 (cattle), 0.999	108.7 (cattle), 107.8 (buffalo), 94.7 (pork)	Model mixtures (0.1, 1, 10%; w/w)	N.I. (−14.1 - +15.3	N.I. (0.61 - 19.40)	N.T.	TaqMan Tetraplex	Hossain et al. (2017)
9	Brazil nut	100 ng - 10 pg, 5 points	3	0.998 - 0.999	84.5 - 93	Model mixtures (100, 1000, 5000, 10000, 100000 mg/kg)	Comparison of true and recovery values at the 95% confidence level	Comparison of intraday and interday (3) RSDr at the 95% confidence level	N.T.	TaqMan Single	de la Cruz et al. (2013)
10	Wheat	1 ng - 0.1 pg, 6 points	N.I.	0.999	96	Commercial food products	N.T.	N.I. (3.41 - 11.01)	N.T.	SYBR Green Single	Mujico et al. (2011)

a. Suggested acceptable parameters and experimental results in parenthesis.
N.I., Acceptable parameters are not indicated; N.T., Not Tested.

1.6.2.2 Amplification efficiency

The amplification efficiency can be calculated from the slope of a standard curve, using the equation: $E = [(10^{(-1/-slope)} - 1] \times 100$. The theoretical maximum E is 100% (slope of −3.322); however, in practice, this parameter was acceptable in the range 90% to 110%, corresponding to a slope of −3.6 to −3.1 (Table 1.4). As discussed in Section 1.4, PCR efficiency can be used to estimate the presence of PCR inhibitors that lead to an increase in Cq values and therefore a decrease in 3.322 Cq span at higher concentrations. In GMO detection, the slope of the inhibition curve should be in the range of $-3.6 \leq slope \leq -3.1$ (ENGL 2015).

1.6.3 Analytical sensitivity

1.6.3.1 Limit of detection (LOD)

Typically, analytical sensitivity is expressed as the LOD in real-time PCR, with two types of LOD being used according to its definition. First, the LOD is defined as the lowest concentration of DNA that yields a fluorescent signal significantly different from that of the negative control (or non-target DNA control) within certain cut-off Cq values (e.g., 37–40 cycles) as determined by the specificity test (Druml, Grandits, Mayer, Hochegger, & Cichna-Markl, 2015; Druml, Mayer, Cichna-Markl, & Hochegger, 2015; Espineira & Vieites, 2012; Iniesto, et al., 2013; López-Andreo, Lugo, Garrido-Pertierra, Prieto, & Puyet, 2005; Taboada, Sanchez, & Sotelo, 2017) or at a specified level of confidence (Alonso-Rebollo, Ramos-Gomez, Busto, & Ortega, 2017; de la Cruz, López-Calleja, Alcocer, González, Martín, & García, 2013; Herrero, Madrinan, Vieites, & Espineira, 2010; I. M. López-Calleja, de la Cruz, Gonzalez, Garcia, & Martin, 2015; Inés María López-Calleja, de la Cruz, Pegels, González, García, & Martín, 2013). For the latter case, the LOD was determined by the International

Conference on Harmonization guidelines (ICH, 2005) as follows:

$$LOD = t \times sd / m,$$

where *sd* is the standard deviation of the negative control, *m* is the slope of the standard curve, and $t = 3.3$ is the Student's *t* for a 95% confidence level.

However, this definition, originally reported for chemical methods, is not perfectly suited for real-time PCR methods, because the Cq value in the negative control (e.g., where the template concentration is zero) cannot be defined (Burns & Valdivia, 2008). In practice, a more popular definition for the LOD is the lowest concentration of DNA at which 95% of the positive simples can be detected, ensuring no more than 5% false negative results (Bustin, et al., 2009; ENGL, 2015). In qualitative GMO detection, the LOD is well defined with LOD_6 and/or $LOD_{95\%}$ methods being recommended (Broeders, et al., 2014). However, there is no general agreement regarding technical standards for food analysis. In most previous studies, the absolute or relative LOD was determined experimentally from the linear dynamic range of the standard curve using three to sixteen replicates (Table 1.4). For further confidence of the relative LOD, DNA extracted from model mixtures (e.g., raw or heat-treated binary mixtures), spiked with certain percentages of target species (e.g., 0.01%, 0.1%, and 1%, w/w), was tested using ten to twenty replicates (Druml, Grandits, Mayer, Hochegger, & Cichna-Markl, 2015; Druml, Kaltenbrunner, Hochegger, & Cichna-Markl, 2016; Druml, Mayer, Cichna-Markl, & Hochegger, 2015; Magdalena Fuchs, Cichna-Markl, & Hochegger, 2012; M. Fuchs, Cichna-Markl, & Hochegger, 2013; Palle-Reisch, Hochegger, & Cichna-Markl, 2015). It should be noted that the theoretical LOD cannot be lower than three copies per PCR, assuming a Poisson distribution and a 95% chance of including single-copy detection; thus, an experimental LOD less than theoretic one is meaningless (Bustin, et al., 2009).

1.6.3.2 Limit of quantification (LOQ)

The LOQ is necessary for the development of reliable quantitative real-time PCR methods. In practice, the LOQ can be determined experimentally from the linear dynamic range of a standard curve or replicates of spiked samples along with the LOD determination, as discussed above. The LOQ is defined as the lowest amount or concentration of analyte that can be reliably quantified with an acceptable level of trueness and precision (ENGL, 2015). Thus, the LOQ cannot be lower than the lowest concentration of the dynamic range (e.g., LOD). In practice, the LOQ has been calculated as follows: (1) $10 \times sb / m$, where *sb* is the standard deviation of the negative control and *m* is the slope of the standard curve (Alonso-Rebollo, Ramos-Gomez, Busto, & Ortega, 2017), or (2) $m\text{Cq} - (2 \times sd)$, where *m*Cq is the mean Cq values of the LOD and *sd* is the corresponding standard deviation (Ferreira, Farah, Oliveira, Lima, Vitorio, & Oliveira, 2016). However, the coefficient of variation (CV, ratio of standard deviation to mean) within 25–30% has been generally used as an acceptable criterion for food analysis (Eugster, Ruf, Rentsch, Hübner, & Köppel, 2008). Specifically, the CV values should be calculated using the variance in copy numbers or concentrations (e.g., % or ng), owing to the logarithmic nature of Cq values (Bustin, et al., 2009).

1.7 Quantification strategy

In real-time PCR methods, the strategies for quantification of certain species used in processed food can be classified by normalization methods into three groups. In the first strategy, the amount of target species DNA in an unknown sample is simply determined by extrapolating the Cq values obtained for the unknown sample in the corresponding standard curve as follows: concentration (or copy number) of species-specific DNA = $10^{[(Cq-b)/a]}$, where a and b represent the slope and the intercept of the standard curve, respectively (Alonso-Rebollo, Ramos-Gomez, Busto, & Ortega, 2017; Mujico, Lombardía, Mena, Méndez, & Albar, 2011; Scarafoni, Ronchi, & Duranti, 2009). In particular, a the ten-fold difference was reported in quantitative results between low-processed and highly processed meat products (Laube, Zagon, & Broll, 2007). Therefore, raw and heat-treated binary mixtures containing known amounts of target material have generally been used for standard curves that can suggest simple relative amount (%) of target DNA (de la Cruz, López-Calleja, Alcocer, González, Martín, & García, 2013; López-Andreo, Aldeguer, Guillén, Gabaldón, & Puyet, 2012; I. M. López-Calleja, de la Cruz, Gonzalez, Garcia, & Martin, 2015; Inés María López-Calleja, de la Cruz, Pegels, González, García, & Martín, 2013). In real-time PCR methods, mitochondrial or chloroplast DNA is the most preferred target sequence for species identification in food samples, as discussed in Section 1.5. However, the amount of these genes varies in different tissues and species; for example, in the case of meat products, equal amounts of beef and pork lean muscle may not contain the same copy numbers of DNA (Ballin, Vogensen, & Karlsson, 2009; López-Andreo, Aldeguer, Guillén, Gabaldón, & Puyet, 2012). This can lead to biased results generated by the group I quantification method, unless sample origin and composition are similar to those of the standard curve.

Therefore, in the second strategy, matrix-adapted standards that were constructed by actual composition and process were used to compensate for this potential inaccuracy. In quantification studies of beef, pork, chicken, and turkey in sausages, the use of matrix-adapted calibrators showed higher precision and accuracy compared with calibration using DNA dilutions from muscle tissues, regardless of PCR (either multiplex or single real-time PCR) and DNA extraction methods used (Eugster, Ruf, Rentsch, Hübner, & Köppel, 2008; Eugster, Ruf, Rentsch, & Köppel, 2009). Moreover, a recent study reported that a calibration mixture containing the proportion of target animal species (e.g., fallow deer) as close as possible to that of samples should be considered necessary for obtaining accurate quantitative results (Kaltenbrunner, Hochegger, & Cichna-Markl, 2018). However, different matrix-adapted reference materials must be prepared for the accurate quantification of each sample type, which would increase the cost and time of assays. To simplify the experimental procedure, Lopez-Andreo et al. (2012) introduced a single-point matrix standard strategy. In this strategy, the standard curves plotted using DNA dilutions from pure beef and pork meat, respectively, are combined with the Cq value obtained using a single matrix reference material (beef:pork, 50:50; w/w) to calibrate the differences (D value) in DNA content for equal amounts of beef and pork meat. This D value is then used to obtain the proportions of pork meat in raw and heat-treated binary mixtures (beef and pork). The accuracy (13–17%) observed was similar to those obtained using the matrix-adapted standards (Eugster, Ruf, Rentsch, & Köppel, 2009).

In the third strategy, reference genes, discussed in Section 1.4, are used to normalize the results obtained from species-specific assays, which can improve the quantification of DNA from different species in food products. In the first normalization method, two standard curves are constructed using a reference (e.g., myostatin) and species-specific (e.g., deer, beef, and pork) systems, respectively, and the amounts of total and species-specific DNA in

samples are determined by each standard curve, as described in the first quantification strategy. The relative quantity of target species is then calculated as follows: target species content (%) = concentration (or copy number) of species-specific DNA/concentration (or copy number) of total DNA × 100. This method has been successfully used to quantify four deer (roe, fallow, red, and sika) species, beef, pork, and horse fractions in various commercial meat products (Druml, Grandits, Mayer, Hochegger, & Cichna-Markl, 2015; Druml, Mayer, Cichna-Markl, & Hochegger, 2015; Iwobi, Sebah, Kraemer, Losher, Fischer, Busch, et al., 2015; Iwobi, Sebah, Spielmann, Maggipinto, Schrempp, Kraemer, et al., 2017). In these studies, single-copy species-specific (i.e., epidermal growth factor pseudogene, lactoferrin, cyclic GMP phosphodiesterase, beta-actin, and growth hormone receptor) and reference (myostatin) genes were used to obtain comparable quantitative results from different species. However, the use of single-copy genes can reduce the sensitivity of real-time PCR assays, limiting their application to various processed food products. Thus, considerable research efforts using multi-copy DNA targets have been expended with the aim to improve the sensitivity and quantitativeness of the resulting measurements.

In the second normalization method for this strategy, a multi-copy reference gene (e.g., 18S rRNA) is used to normalize the Cq values of samples. The standard curves are plotted using DNA extracted from raw and heat-treated binary mixtures containing different percentages of target species, and the Cq values obtained for each sample using the species-specific systems (CqSP) are normalized as follows: CqSPS = CqEU × CqSP / CqEUS, where CqSPS is the normalized Cq value of the sample analyzed using the species-specific systems, CqEU is the average Cq value obtained from the standard samples (binary mixtures) using the reference system, and CqEUS is the Cq value of the sample analyzed using the reference system (Fajardo, et al., 2008). The amount of target species DNA in an unknown sample is measured by the normalized Cq values of the standard curve, as described in the first

quantification strategy. This method has been successfully used for the relative quantification of three deer (red, fallow, roe) species, pork, and seven game birds (quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, and song thrush) fractions in various commercial meat products (Fajardo, et al., 2008; M. Kim, Yoo, Lee, Hong, & Kim, 2016; Rojas, Gonzalez, Pavon, Pegels, Lago, Hernandez, et al., 2010). In these studies, multi-copy species specific (mitochondrial D-loop and 12S rRNA) and reference (18S rRNA) genes were used, showing improved LOQ for pork (0.1% vs. 1%) and roe deer (0.00004% vs. 0.5%) detection, as compared with the results of the single-copy systems discussed for the above products (Druml, Mayer, Cichna-Markl, & Hochegger, 2015; Iwobi, et al., 2015).

In the third normalization method, similar to the second method, the 18S rRNA gene is used to generate the ΔCq values, which are calculated as follows: $\Delta Cq = Cq_{target} - Cq_{reference}$, where Cq_{target} and $Cq_{reference}$ are the Cq values obtained using species-specific and reference systems, respectively. The normalized standard curves are plotted using the calculated ΔCq values and DNA extracted from raw and heat-treated binary mixtures containing different percentages of target species. The target DNA in an unknown sample is measured by the normalized standard curves as for other quantification methods (Soares, Amaral, Oliveira, & Mafra, 2013). This approach is one of the most popular normalization methods for relative quantification. Whereas the first and second normalization methods have been mostly used for the quantification of meat products, the third method has been widely applied to quantify meat (horse and pork) as well as plant (safflower and soybean) species in various commercial products (Amaral, Santos, Oliveira, & Mafra, 2017; J. Costa, Amaral, Grazina, Oliveira, & Mafra, 2017; Meira, Costa, Villa, Ramos, Oliveira, & Mafra, 2017; Soares, Amaral, Oliveira, & Mafra, 2013; Villa, Costa, Oliveira, & Mafra, 2017).

It should be noted that, for accurate normalized quantification, the amplification efficiency of the reference system is almost comparable with that of the species-specific

system. For example, in the relative quantification of deer species in heat-treated products, the difference in size between the myostatin reference (97 bp) and species-specific systems (62 bp and 68 bp) substantially led to overestimated recoveries (> 100%), mostly owing to the increased Cq values in the reference system (Druml, Kaltenbrunner, Hochegger, & Cichna-Markl, 2016). Alternatively, the use of a novel myostatin reference system (70 bp) improved the amplification efficiency and therefore showed lower bias in quantification of heat-treated products (Druml, Kaltenbrunner, Hochegger, & Cichna-Markl, 2016).

1.8. Method validation

1.8.1 Trueness

In quantitative measurements, the trueness is also referred to as accuracy and is defined as the closeness of agreement between the estimated value and the true value (or accepted reference value). The trueness is usually expressed in terms of bias [or systemic error (%) = (mean estimated value - true value) / true value × 100)]. In GMO detection, the trueness should be within 25% of the accepted reference value (ENGL, 2015). However, unlike GMO testing, there is no certified reference material for food analysis, and therefore in-house reference mixtures containing certain percentages of target species are usually used for the trueness of real-time PCR assays. Generally, bias within 25–30% has been considered as an acceptable criterion in previous real-time PCR studies for food analysis (Table 1.4).

1.8.2 Precision

Precision is defined as the degree of agreement of measurements under specified conditions, and in its practical use, it can be divided into two groups: repeatability (or short-term precision) for intra-laboratory variation and reproducibility (or long-term precision) for inter-laboratory variation. The latter is usually defined by collaborative studies (ENGL, 2015; Kralik & Ricchi, 2017). Thus, this section only focuses on the repeatability as an indicator of the precision of a real-time PCR method. In quantitative real-time PCR methods, the repeatability is defined as the closeness of agreement between successive and independent results that are obtained with the same method, on identical test items, in the same laboratory, by the same operator, and using the same equipment within short intervals of time (ENGL, 2015). Generally, repeatability is expressed as the relative standard deviation (RSDr) of test results and consists of intra- and inter-day variation. The former indicates the variation of the

replicates analyzed in the same experiment, whereas the latter describes the variation between different experiments conducted on different days under repeatability conditions (Kralik & Ricchi, 2017). In food analysis by real-time PCR assays, the repeatability of standard curves has been evaluated by intra- and inter-assay (two to five consecutive days) variation (Druml, Grandits, Mayer, Hochegger, & Cichna-Markl, 2015; Druml, Hochegger, & Cichna-Markl, 2015; Druml, Mayer, Cichna-Markl, & Hochegger, 2015). In practice, the popular approach is to test the intra- and/or inter-day (two to seven days) repeatability using in-house reference mixtures containing certain levels (e.g., near to the LOD) of target species, as for trueness (Table 1.4). In GMO detection, RSDr (CV, %) should be ≤ 25% as established on samples containing 0.1% GM related to the mass fraction of GM material and with a sufficient number of test results, at least 15 (ENGL, 2015). In food analysis, there are no specific recommendations for RSDr, spiking levels, and replicate numbers; however, most previous studies considered CV within 25% established using three to nine repeats as an acceptable criterion of the repeatability (Table 1.4). As discussed for LOQ, Cq values should not be used to calculate CV values for repeatability evaluation (Bustin, et al., 2009).

1.8.3 Robustness

The robustness of a quantitative or qualitative real-time PCR method constitutes the degree of unaffectedness by slightly different experimental conditions that may impact on the results. The factors that should be tested and acceptable criteria in GMO detection are well documented in the articles by ENGL, (2015) and Broeders et al. (2014). An acceptable value should be ≤ 30% as a combination of trueness and repeatability for quantitative analysis, and the qualitative method should detect the positive results in terms of presence of the target. As for the previous measures discussed in this review, there are no specific criteria of the robustness in real-time PCR methods for food analysis. However, evaluation of the use of

two to three different DNA extraction methods, real-time PCR instruments, master mixes, annealing temperatures (± 1°C), and/or reaction volumes (± 5%) for the robustness of real-time PCR methods in previous studies usually suggested no significantly different results or detection of the target (D'Andrea, Coïsson, Locatelli, Garino, Cereti, & Arlorio, 2011; Druml, Grandits, Mayer, Hochegger, & Cichna-Markl, 2015; Druml, Hochegger, & Cichna-Markl, 2015; Druml, Mayer, Cichna-Markl, & Hochegger, 2015; Magdalena Fuchs, Cichna-Markl, & Hochegger, 2012; Iwobi, et al., 2015; Kaltenbrunner, Hochegger, & Cichna-Markl, 2018).

1.9 Conclusions

With expansion of the processed-food market and global trading of food products, the development of accurate and rapid methods of analysis is a critical component of food safety. Real-time PCR constitutes an emerging technique for the identification and authentication of food products, with many research groups having developed their own in-house-validated methods. However, the lack of standard parameters for the development and validation of real-time PCR methods can lead to inconsistent results upon their application to different laboratories and conditions. This study reviewed different methods and parameters scattered throughout a large number of scientific papers and suggested integral principles, from basic DNA extraction to advanced quantification methods, along with acceptable criteria required for the in-house validation of real-time PCR methods in food analysis. Specially, this study provided an in-depth review of PCR inhibition and amplification controls that are essential for reliable real-time PCR results. For the first time, different quantification strategies were compared and assessed, which will lead to the improvement of quantification accuracy in food adulteration assays. Therefore, this review will provide researchers with a beneficial guide for development of real-time PCR methods in a harmonious manner and contribute to an enhanced applicability of the developed methods.

Chapter 2[ii]

A comparative study of quantitative real-time PCR methods for the pork meat adulteration in processed beef products

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2.1 Abstract

Meat products are a main protein source, and therefore adulterated meat products have raised concerns for health and food safety as well as religious beliefs. Quantitative real-time PCR (qPCR) is a modern technique that has been widely used for the detection of species used in meat products. It can play an integral role to guide whether the detected adulteration originates from unintentional cross-contamination or purposeful substitution. For accurate and reliable qPCR examination, quality and quantity of isolated DNA and quantification approaches of qPCR products are critical. In this study, we assessed two common DNA quantification methods for isolated DNA and five quantification approaches for qPCR products through estimation of pork meat in commercial beef products. Our findings clearly indicated that the spectrofluorometric DNA quantification method and qPCR methods with normalized standard curves using reference systems (e.g., 18*S* rRNA gene) can improve the accurate, reliable quantification for processed meat products.

Keywords: Pork meat adulteration; DNA quantification; 18*S* ribosomal RNA gene; Normalization methods;

2.2 Introduction

Meat is an important food ingredient that has been world-widely used to manufacture numerous types of food products, such as hams, sausages, meatballs, and burger patties. However, the morphological characteristics of meat used in these processed products are easily lost during the manufacturing processes, including mincing, grinding, mixing, and heating. This nature of processing tempts into adulterations in meat products, such as the substitution of a valuable authentic meat with a less expensive ingredient, or the false declaration of either raw material origin or the production process used to manufacture an ingredient. Such fraudulent practices have raised concerns for health and food safety as well as religious beliefs. For instance, the European horsemeat scandal in 2013 clearly demonstrates how adulteration negatively influences global food safety and economy (Moyer, DeVries, & Spink, 2017). Therefore, many, if not all, food authorities reinforce regulatory monitoring of the authenticity of commercial meat products since 2013 (Chuah, He, Effarizah, Syahariza, Shamila-Syuhada, & Rusul, 2016; Kane & Hellberg, 2016; Naaum, Shehata, Chen, Li, Tabujara, Awmack, et al., 2018).

Development of accurate, convenient, reliable detection methods constitutes an important first line of defense for both detecting and deterring food fraud. Various techniques have been successfully applied to identify and quantify meat species, such as liquid chromatographic methods, vibrational spectroscopices, and enzyme linked immunosorbent assays (Giaretta, Di Giuseppe, Lippert, Parente, & Di Maro, 2013; Hu, Zou, Huang, & Lu, 2017; Macedo-Silva, Barbosa, Alkmin, Vaz, Shimokomaki, & Tenuta-Filho, 2000). Among proposed detection methods, DNA-based methods show high stability, efficiency, and accuracy, and play a more important role than other methods. Numerous studies have already reported the applicability of polymerase chain reaction (PCR)-based techniques for the

identification of meat species, including species-specific PCR, PCR-restriction fragment length polymorphism, forensically informative nucleotide sequencing, and DNA barcoding (Kane & Hellberg, 2016; Kitano, Umetsu, Tian, & Osawa, 2007; Lee, Kim, Jo, Jung, Kwon, & Kang, 2016; Maede, 2006; Naaum, et al., 2018). These methods are essentially qualitative methods that can efficiently identify animal species present or absent in meat products, but they are not constructed to quantify amounts of meat species. In practice, processed meat products can be unintentionally cross-contaminated with trace amounts of other meat species during manufacturing processes, leading to undeclared species detection by the qualitative methods. Therefore, in meat product analysis, quantification is a must to guide whether this mislabeling is caused by unintentional cross-contamination or purposeful substitution. In this regard, real-time PCR is an alternative technique that can allow not only the identification but also the quantification of species used in meat products based on the presence of genomic DNA. Extensive studies have been conducted to develop quantitative real-time PCR (qPCR) for the authentication of meat products. However, quantifying the amounts of certain species used in meat products is still challenging, mostly owing to differences in DNA extractability, DNA degradation, species genome size, tissue cell size, and mitochondrial distribution (Ballin, Vogensen, & Karlsson, 2009).

In order to overcome these limitations in quantification, considerable attentions should be given specifically to two steps of qPCR methods: quantification of DNA isolated from the examined specimens, and determination of DNA quantities in qPCR amplification. Isolated DNA from the examined sample is typically quantified by either spectrophotometric or spectrofluorometric method, with the former representing the most commonly used technique. While it is common, spectrophotometry has critical limitations, including sensitivity to single stranded DNA, RNA, proteins, and organic contaminants (such as chloroform and phenol) commonly found in isolated DNA (J. Costa, Amaral, Grazina,

Oliveira, & Mafra, 2017; Scarafoni, Ronchi, & Duranti, 2009). They can lead to over- or underestimation of quantity, resulting in inaccuracy of genomic DNA amount in the isolated DNA samples. In contrast, the spectrofluorometric method uses fluorescent dyes specific to double-stranded DNA (dsDNA), the fluorescence of which is measured and used to calculate the amount of dsDNA present in the DNA extract (Yalcinkaya, Yumbul, Mozioglu, & Akgoz, 2017). The quantification of amplicons in qPCR is the other critical factor in qPCR meat authentication. It depends on the choice of standard curve and its preparation. There are three groups by quantification approaches based on 1) absolute standard curve, 2) relative standard curve, and 3) normalized standard curve. In the first approach, standard curves are plotted using quantification cycle (Cq) values and the logarithm of DNA dilutions from muscle tissue of the target meat species (Hossain, et al., 2017). The DNA concentration in samples is simply determined by extrapolation. In the second approach, matrix-adapted standards that are constructed by actual composition and process of samples are generally used for the calibration, leading to significant improvement in precision and accuracy (Eugster, Ruf, Rentsch, Hübner, & Köppel, 2008; Eugster, Ruf, Rentsch, & Köppel, 2009). In the third approach, reference genes (e.g., 18*S* ribosomal RNA and myostatin) are used to normalize the results obtained from species-specific assays, which can improve the quantification of DNA from different species in food products. (Amaral, Santos, Oliveira, & Mafra, 2017; Druml, Grandits, Mayer, Hochegger, & Cichna-Markl, 2015; Druml, Mayer, Cichna-Markl, & Hochegger, 2015; Fajardo, et al., 2008; Iwobi, et al., 2015; Iwobi, et al., 2017; M. Kim, Yoo, Lee, Hong, & Kim, 2016; Meira, Costa, Villa, Ramos, Oliveira, & Mafra, 2017; Rojas, et al., 2010; Soares, Amaral, Oliveira, & Mafra, 2013). Based on these three approaches, currently five different types of quantification methods have been commonly used.

In the present study, we aim to compare these common methods of qPCR authentication to determine the best quantification approach. To the best of our knowledge,

this is the first study to compare and evaluate various quantification methods for obtaining accurate and reliable qPCR results. The spectrophotometric and spectrofluorometric methods were assessed by comparing the quantity and purity of the DNA extracts as well as by analyzing acceptable parameters of qPCR results. Pork-specific and reference (18*S* rRNA) systems were employed for qPCR, and their amplification efficiency was compared. Standard curves were constructed for five different quantification methods, and the accuracy and precision of each method were further validated using in-house model mixtures. Finally, the validated methods were applied to fourteen commercial beef products, and the pork meat adulteration was quantitatively assessed to confirm our observations.

2.3 Materials and methods

2.3.1 Samples and model mixtures

Fresh lean meat samples of beef (*Bos taurus*) and pork (*Sus scrofa*) were purchased from local butchers in Saskatoon city of Canada and used as reference materials for the validation of qPCR assays.

For standard curve preparations, reference binary mixtures (10%, 1%, 0.1%, 0.01%, and 0.001%; w/w) were prepared. Pork and beef meat was separately minced with a food processer, and the initial binary mixture (10%) was prepared by adding 10 g of minced pork meat into 90 g of minced beef meat. The subsequent mixtures were prepared by ten-fold serial dilutions using minced beef meat. For the method validation and the comparison of quantitative results, the pork model mixtures containing 50%, 5%, 2.5%, and 0.25% (w/w) of pork meat were prepared similar to the binary mixtures. In-house model burger was also prepared by mixing ground lean beef (670 g), ground pork (750 g), mushroom (200 g), carrot (100 g), garlic (60 g), chili pepper (80 g), onion (400 g), and breadcrumb (150 g), and appropriate amounts of seasonings, including sugar, salt, soybean source, black pepper, sesame oil, and cooking wine. All samples (binary mixtures, model mixtures, model burger, and pure beef and pork meat) used for qPCR assays were subjected to heat treatment at 121℃ for 15 min for the effect of thermal processing, which is usually applied to commercially processed meat products.

As the real specimens of commercial meat products, a total of 14 different commercially available beef products, including ground beef (n = 1), seasoned roast beef (n=3), jerky (n = 1), sausages (n = 3), meatball (n = 2), burger (n = 1), and pressed ham (n=3), were purchased from local markets in Saskatoon city of Canada. Since DNA extraction and amplification from commercial beef products can be hampered by inhibitory substances in

commercial products, such as preservatives, seasonings and/or spices, the commercial samples were washed well with distilled water, as described previously by Kim et al. (2018).

2.3.2 DNA extraction

DNA was extracted from 30 mg of meat selected from each sample using the EZ-10 Spin Column Genomic DNA Minipreps kit (Bio-Basic Inc., Markham, ON, Canada), according to the manufacturer instructions. Briefly, the samples were mixed with 300 µL ACL solution and 20 µL protease K, and the mixture was incubated at 55℃ until complete lysis. Additionally, 20 µL RNase A (10 mg/ml) was added to the mixture, and it was incubated at room temperature for 5 min for obtaining RNA-free genomic DNA. The lysis solution was then mixed with 300 µL AB solution, and the lysate was transferred to an EZ-10 Spin column. After centrifugation at 2000 × *g* for 2 min, the column was washed twice with 500 µL washing buffer, and purified DNA was eluted by adding 50 µL elution buffer.

2.3.3 DNA quantification

2.3.3.1 Spectrophotometric method

The extracted total DNA was quantified using the NanoDrop ND-2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA concentrations were determined by measuring UV absorbance at 260 nm (1 absorbance unit corresponds to 50 µg/mL dsDNA). The purity of the extract was determined by calculating the ratios of absorbance measured at 260/280 nm and at 260/230 nm.

2.3.3.2 Spectrofluorometric method

To represent spectrofluorometric methods of dsDNA quantification, Quant-iT

Picogreen dsDNA assay kit (Invitrogen, Eugene, OR, USA) was used according to the manufacturer instructions. Briefly, the lambda DNA, quantified at concentrations of 1000, 500, 250, 100, 50, 10 ng/ml, was used to make standard curve. DNA extrated from samples was diluted 1:100 in TE buffer to a final volume of 1 ml. This DNA dilution was mixed with 1 ml of Quant-iT PicoGreen reagent solution in disposable cuvettes (Bio-Rad, Hercules, CA, USA) and then incubated for 5 min at room temperature. The fluorescence of the mixture was measured using a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) with an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The DNA concentration of an unknown sample was determined from the standard curve.

2.3.4 Primer sets

Species-specific primer sets used in this study targeted short fragments of cytochrome b (*cytb*) genes of beef (Bos-F 5'- CTGCCGAGACGTGAACTACG-3', Bos-R: 5'-AAGCCTCGTCCTACGTGCATAT-3', 99 bp) and pork (Prk-F: 5'-CTGCCCTGAGGACAAATATCATTC-3', Prk-R 5'- AAGCCCCCTCAGATTCATTCTACG-3', 107 bp) (Amaral, Santos, Melo, Oliveira, & Mafra, 2014; Amaral, Santos, Oliveira, & Mafra, 2017). A reference system, 18*S* ribosomal RNA (18*S* rRNA) gene (18SRG-F: 5'-CTGCCCTATCAACTTTCGATGGTA-3', 18SRG-R: 5'-TTGGATGTGGTAGCCGTTTCTCA-3', 113 bp), was used as an external control to assess the amplifiability of DNA extracts and false negative results of the qPCR assays (Joana Costa, Oliveira, & Mafra, 2013). Additionally, this reference system was used to normalize the results obtained using the species-specific systems for quantitative analysis.

2.3.5 qPCR conditions

qPCR analysis was performed in a total volume of 20 µL, containing 2 µL (10 ng) of

template DNA, 0.25 μM of each primer, 10 μL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), and sterile distilled water. The reactions were performed using CFX96 real-time detection system (Bio-Rad) under the following conditions: 98 ℃ for 3 min followed by 30 cycles of 98℃ for 10 s and 60℃ for 15 s. Amplification was followed by a melt-curve analysis between 65℃ and 95℃ using a 0.5℃ increment. A no-template control was used for the negative control PCR, and PCR specificity and product detection were verified by examining the temperature-dependent melting curves of the PCR products and ethidium bromide staining on 2% agarose gel.

2.3.6 Validation of the qPCR system

In qPCR assays, standard curves can act as a simple, rapid, and reproducible indicator for evaluating the amplification efficiency and analytical sensitivity. Thus, following criteria were used to define an acceptable qPCR assay, as previously described (Bustin, et al., 2009; ENGL, 2015).

2.3.6.1 Amplification efficiency

The linear dynamic range should ideally extend over four $\log_{10}$ concentrations, the coefficient of determination (R^2) should be over 0.98, and the amplification efficiency, which is calculated using the equation: $E = [(10^{(-1/\text{-slope})}\text{-}1] \times 100$, should be in the range of 110% to 90%, corresponding to a slope between -3.1 to -3.6.

2.3.6.2 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD is defined as the lowest concentration of DNA at which 95% of the positive simples can be detected under 30-cycle conditions, ensuring no more than 5% false negative results. The LOQ is defined as the lowest amount or concentration of analyte that

can be reliably quantified with an acceptable level of trueness and precision. Generally, the relative standard deviation (RSD; ratio of standard deviation to mean) under the repeatability conditions should be in the range of 25% for quantitative food analysis.

2.3.6.3 Trueness and repeatability

The trueness is defined as the closeness of agreement between the estimated value and the true value, and usually expressed as the bias (mean estimated value - true value) / true value × 100). The repeatability of an assay is defined as the RSD of test results under the repeatability conditions. As acceptance criterion, these two parameters should be within the 25% range.

2.3.7 Quantification methods

For obtaining accurate and reliable results by the qPCR assays, five different quantification approaches were independently employed.

2.3.7.1 Method #1

The absolute standard curve for the pork-specific qPCR system was plotted using four-fold serially diluted DNA (5–0.005 ng/µL) extracted from heat-treated pure pork meat (Hossain, et al., 2017). The quantity of pork DNA in an unknown sample was simply determined by extrapolating the Cq values obtained for the unknown sample in the corresponding standard curve as follows: concentration of pork DNA = $10^{[(Cq - b)/a]}$, where a and b represent the slope and the intercept of the standard curve, respectively. This basic principle was applied to the quantification Methods #2 – #5 below. The relative quantity of pork DNA was simply calculated as follows: pork content (%) = DNA concentration estimated by pork-specific qPCR / total DNA concentration (10 ng) used in pork-specific

qPCR × 100.

2.3.7.2 Method #2

This method proposed by Druml, Mayer et al. (2015) employs a reference qPCR system for the normalization of results; thus, in this study, we classified it into the third quantification approach, as described in the Introduction. Two absolute standard curves for the reference (e.g., 18S rRNA) and pork-specific systems were constructed by analyzing serially diluted DNA extracted from a heat-treated 50%-pork model mixture. DNA extract was adjusted at a concentration of 10 ng/µL and then serially diluted with distilled water in four-fold steps. The concentration of total meat DNA ranged from 0.01 to10 ng/µL, and thus the concentration of pork DNA was in the range of 0.005 to 5 ng/µL. The concentrations of pork and total meat DNA in samples were determined using each standard curve. The relative quantity of pork DNA was calculated as follows: pork content (%) = concentration of pork DNA / concentration of total DNA × 100.

2.3.7.3 Method #3

This method followed the approach described by Pegels et al. (2011). The relative standard curve for the pork-specific system was plotted using DNA extracts (5 ng/µL) from the reference binary mixtures (100–0.001%). This relative standard curve was further normalized in the quantificaiton Methods #4 and #5 below.

2.3.7.4 Method #4

Procedures propsoed by Fajardo et al. (2008) was employed for Method #4. The Cq values obtained for each sample using the pork system (CqSP) were normalized as follows: CqSPS = CqEU × CqSP / CqEUS, where CqSPS is the normalized Cq value of the sample

analyzed using the pork system, CqEU is the average Cq value obtained for the binary mixtures using the reference system, and CqEUS is the Cq value of the sample analyzed using the reference system. The normalized standard curve was plotted using the calculated CqSPS values and the pork DNA in an unknown sample was measured using the normalized standard.

2.3.7.5 Method #5

This method was proposed by Soares et al. (2013). The ΔCq values normalized by the reference system were calculated as follows: $\Delta Cq = Cq_{pork} - Cq_{reference}$, where Cq_{pork} and $Cq_{reference}$ are the Cq values obtained using the pork and and reference systems, respectively. The normalized standard curve was plotted using the calculated ΔCq values and the pork DNA in an unknown sample was measured using the normalized standard.

2.4 Results

2.4.1 Specificity of primer sets

The specificity of primer sets used in this study has been already tested via the *in silico* and *in suit* analyses, previously demonstrating no cross-reactivity (Amaral, Santos, Melo, Oliveira, & Mafra, 2014; Amaral, Santos, Oliveira, & Mafra, 2017; Joana Costa, Oliveira, & Mafra, 2013). In this study, we tested the specificity of each primer set using the two reference materials only. The beef-specific qPCR assay showed that the average Cq values obtained for pure beef DNA was 14.35 ± 0.24. In the pork-specific system, the average Cq value obtained for pure pork DNA was 15.03 ± 0.11; however, significant signals (31.83 ± 0.29) were observed when beef DNA was analyzed under the 40-cycle conditions. In the case of reference system, the average Cq values obtained for pure beef and pork DNA were 18.24 ± 0.04 and 17.24 ± 0.05, respectively, However, non-specific signals (35.57 ± 0.11) were observed in the negative controls with 40-cycle PCR; thus, PCR cycle was adjusted to the stringent 30-cycle condition. Following qPCR, temperature-dependent melting curves (T_m: 80.5℃, 80.0℃, and 85.0℃ for beef, pork, and reference systems, respectively) were defined to confirm the generation of non-specific signals. The parameters observed under the optimized conditions were comparable to the previous qPCR results obtained using the same primer sets.

2.4.2 Comparison of DNA quantification methods and relative standard curve for Method #3

The quantity and purity of DNA extracts are critical factors dominating qPCR results. In this study, spectrophotometric and spectrofluorometric methods were employed and compared for obtaining accurate and reliable qPCR results. The spectrofluorometric analysis

showed that the average concentration of DNA extracts was 14.51 ± 3.97 ng/μL, which was lower than that (78.38 ± 18.63 ng/μL) quantified by the spectrophotometric method over five times. Our result was in well accordance with the comparison results of DNA extraction methods for meat analysis conducted by Yalcinkaya et al. (2017). The purity of DNA extracts determined by the spectrophotometric method was 1.81 ± 0.04 at 260/280 nm and 1.49 ± 0.29 at 260/230 nm. In, general, values over 1.8 (for $A_{260:280}$) and between 2.0 and 2.2 (for $A_{260:230}$) are considered as pure DNA; however, these values are strongly dependent on the matrices used. When considering the values observed at 260/280 nm and the analytical samples (e.g., heated-treated and processed food) used in this study, DNA extracts were acceptable to be used in qPCR analysis of meat products. Further evaluation of both DNA quantification methods was conducted by comparing quantitative parameters of relative standard curves. The relative standard curves for the pork-specific system were plotted using DNA extracts from the heat-treated reference binary mixtures (10%–0.0001%, w/w). As shown in Figures 2.1A and 2.1B, linear correlation (R^2) and amplification efficiency (E) were 0.994 and 95.53%, respectively, for the spectrophotometric method and 0.998 and 101.01%, respectively, for the spectrofluorometric method, which were in the acceptable range for qPCR. However, the spectrofluorometric method showed better linear dynamic range (100%–0.01%) than that of the spectrophotometric one (100%–0.1%), which is likely overestimation of the spectrophotometric method due to the contaminated single-strand DNA, RNA and proteins. Therefore, the spectrofluorometric DNA quantification method was used for further evaluation of qPCR analyses of meat products.

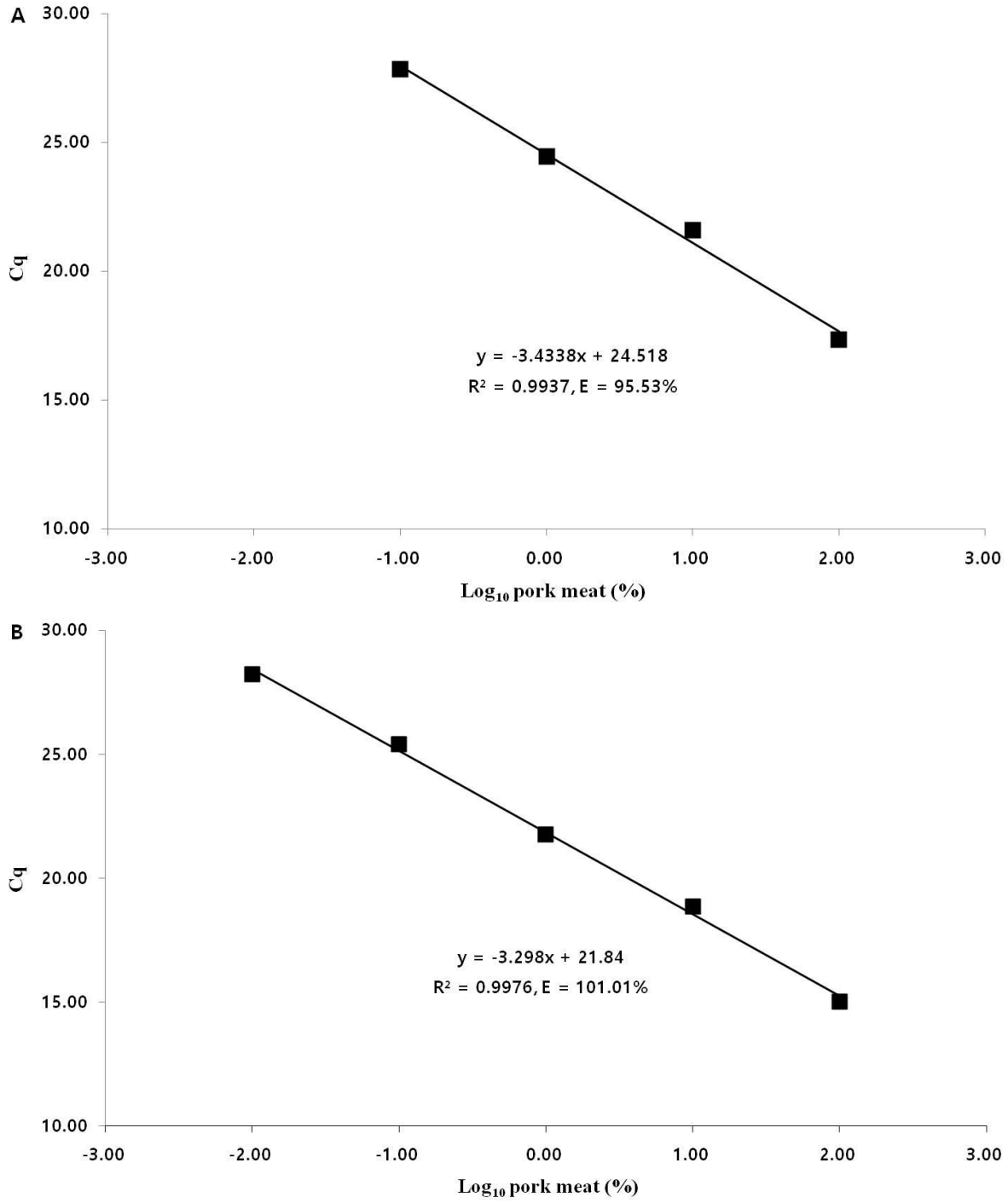
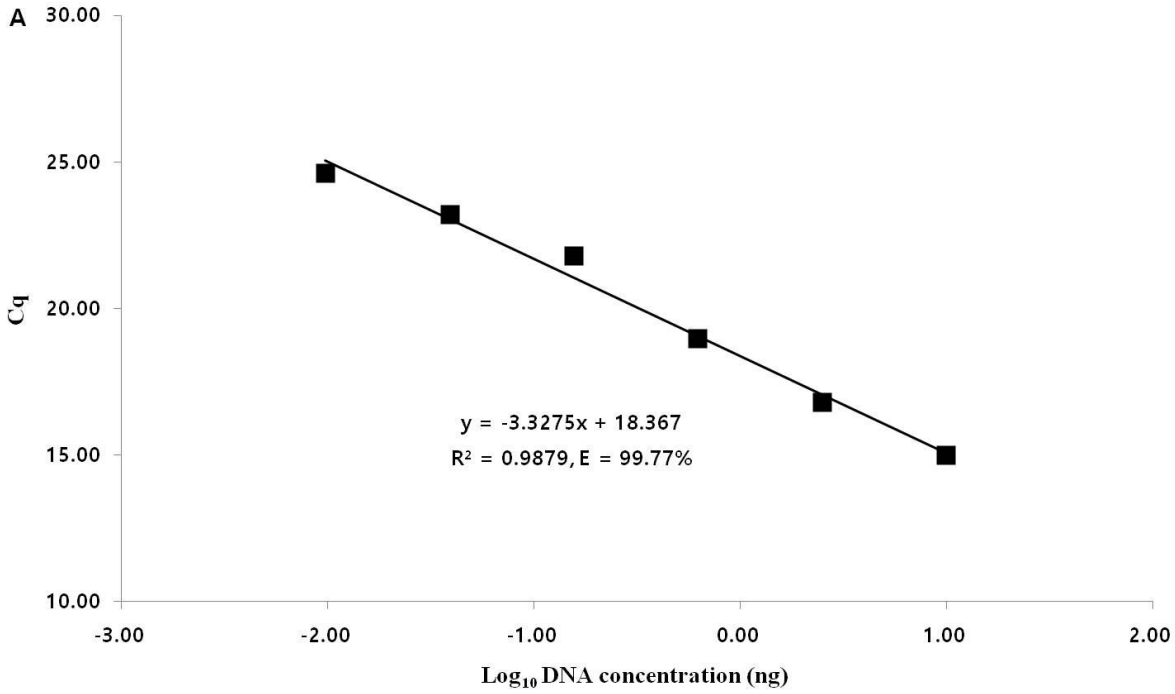


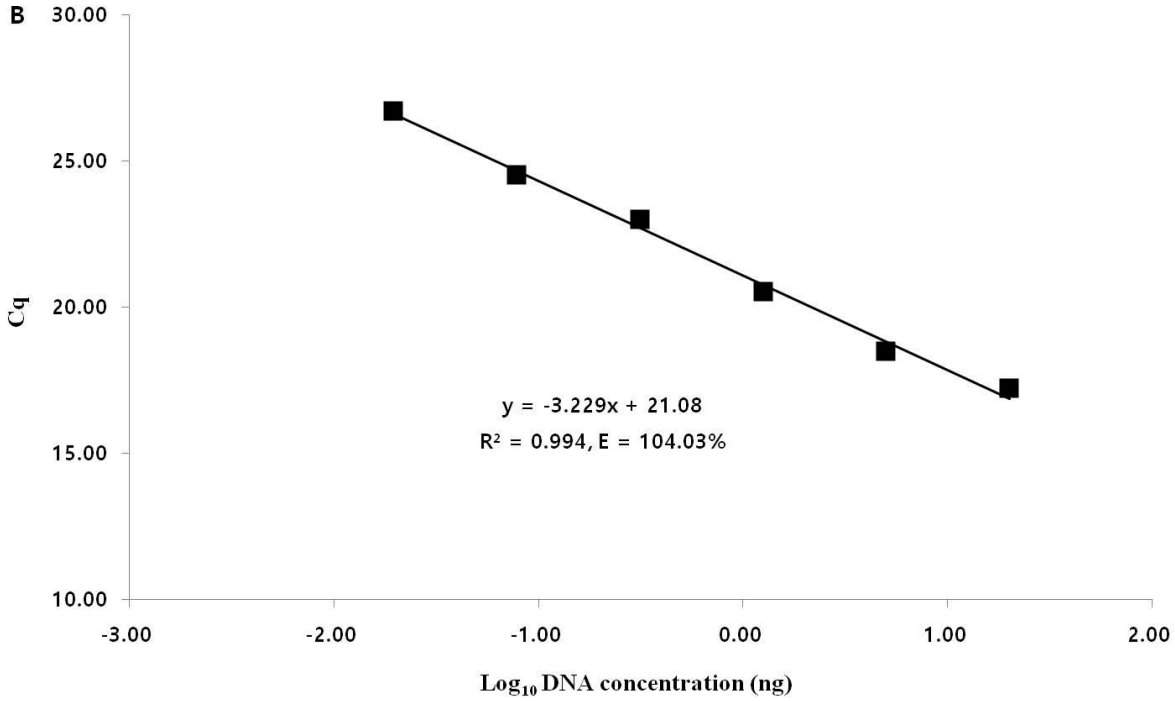
Figure 2.1 Relative standard curves constructed using DNA quantified by the spectrophotometric (A) and spectrofluorometric (B) methods. DNA was independently extracted in triplicate, and the qPCR assays were conducted in triplicate on three different days.

2.4.3 Absolute standard curves for Method #1 and #2

The absolute standard curve for the pork-specific qPCR system for Method #1 was plotted using four-fold serial DNA dilutions (Figure 2.2A). The linear dynamic range was in the range of 5-0.005 ng/µL under which R^2 and E values were 0.988 and 99.77% (corresponding to a slope of -3.327), respectively. For the quantification Method #2, two standard curves for the reference (18*S* rRNA) and pork-specific systems were constructed similar to the absolute standard curve, as described above. In both qPCR assays, R^2 was 0.994, and E for the reference and pork-specific systems was 104.03% and 107.11%, respectively, (Figures 2.2B and 2.2C).

It should be noted that differences in size between reference and species-specific systems could lead to the overestimated recoveries in qPCR, and therefore the amplification efficiency of both systems should be almost comparable for obtaining the accurate qPCR results (Druml, Kaltenbrunner, Hochegger, & Cichna-Markl, 2016). The two primer sets employed in this quantitative study produced the almost same size of amplicon (113 bp for eukayotes and 107 bp for pork); therefore, the equivalent amplification efficiency of the two systems guaranteed their use for further quantitative analysis.


A
30.00
25.00
20.00
15.00
10.00
Cq
y = -3.3275x + 18.367
R² = 0.9879, E = 99.77%
-3.00
-2.00
-1.00
0.00
1.00
2.00
Log10 DNA concentration (ng)


B
30.00
25.00
20.00
15.00
10.00
Cq
y = -3.229x + 21.08
R² = 0.994, E = 104.03%
-3.00
-2.00
-1.00
0.00
1.00
2.00
Log10 DNA concentration (ng)

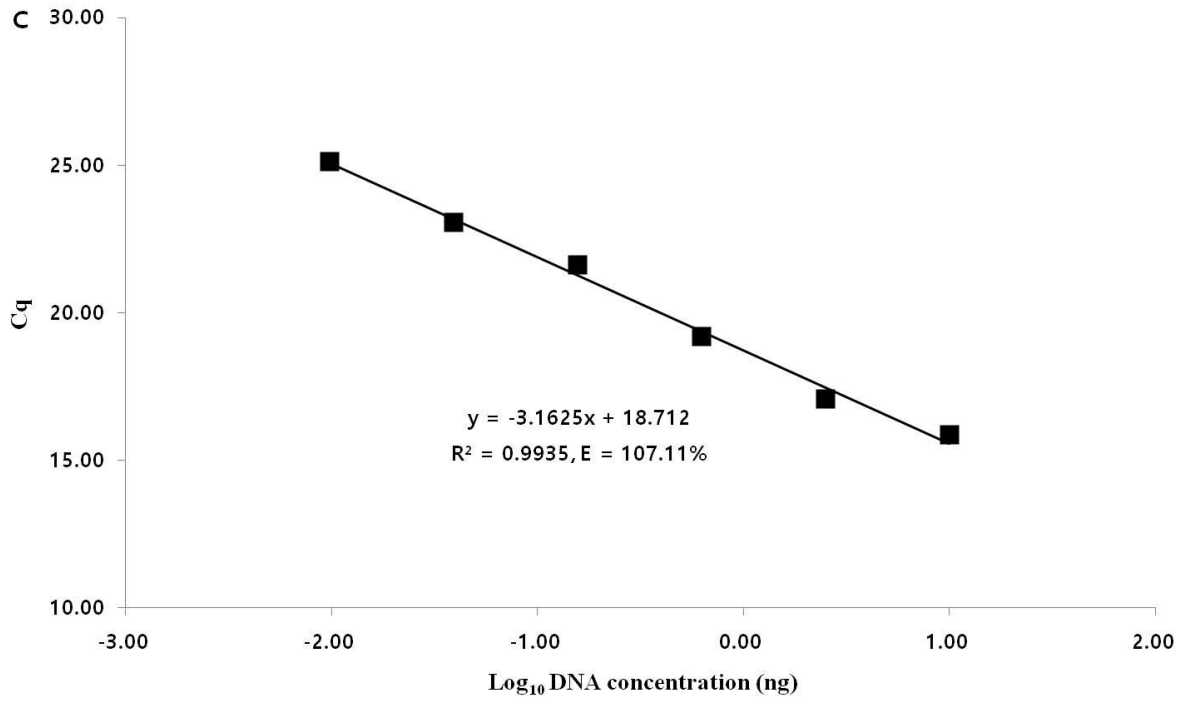


Figure 2.2 Absolute standard curve constructed using DNA dilutions from pure pork meat (A). Two standard curves constructed using DNA dilutions from 50% pork mixture for the reference (B) and pork-specific (C) systems. DNA was independently extracted in triplicate, and the qPCR assays were conducted in triplicate on three different days.

2.4.4 Normalized standard curves for Method #4 and #5

For the quantitative analysis of pork meat in a beef product, the relative standard curve constructed in Section 3.2 was further normalized for Methods #4 and #5 by the Cq values obtained using the reference system, as described in Materials and Methods. The normalized Cq values and average Cq values obtained using the reference system were listed in Table 2.1. The normalized standard curves were plotted using normalized Cq values and the logarithm of pork meat percentages (100, 10, 1, 0.1, and 0.01%). For the standard curve normalized by the quantification Method #4, R^2 and E values were 0.998 and 100.71%, respectively (Figure 2.3A). These values of 0.998 and 101.02%, respectively, were observed for the standard curve normalized using the ΔCq method for Method #5 (Figure 2.3B). As it can be inferred from both normalized standard curves, these parameters could well satisfy acceptable criteria suggested by other documents and thus be used to quantify pork amounts.

Table 2.1 The average and normalized Cq values obtained using pork-specific and reference qPCR systems to create the normalized standard curves.

	Pork (%)	Cq value[a]			Normalized Cq value	
		Pork	Reference (average)		Method 4	Method 5
	100	15.19	17.83		15.12	-2.63
	10	18.93	17.84		18.83	1.09
Day 1	1	21.72	17.86	(17.74)	21.58	3.86
	0.1	25.68	17.71		25.73	7.97
	0.01	28.47	17.49		28.88	10.98
	100	14.93	17.18		15.14	-2.25
	10	18.75	17.76		18.39	0.98
Day 2	1	21.75	17.29	(17.42)	21.92	4.46
	0.1	25.26	17.26		25.49	8.00
	0.01	28.02	17.60		27.73	10.42
	100	14.90	17.27		15.07	-2.37
	10	18.86	17.49		18.83	1.37
Day 3	1	21.75	17.62	(17.46)	21.56	4.13
	0.1	25.21	17.37		25.35	7.84
	0.01	28.19	17.56		28.03	10.63

a. Values are the mean of three replicates.

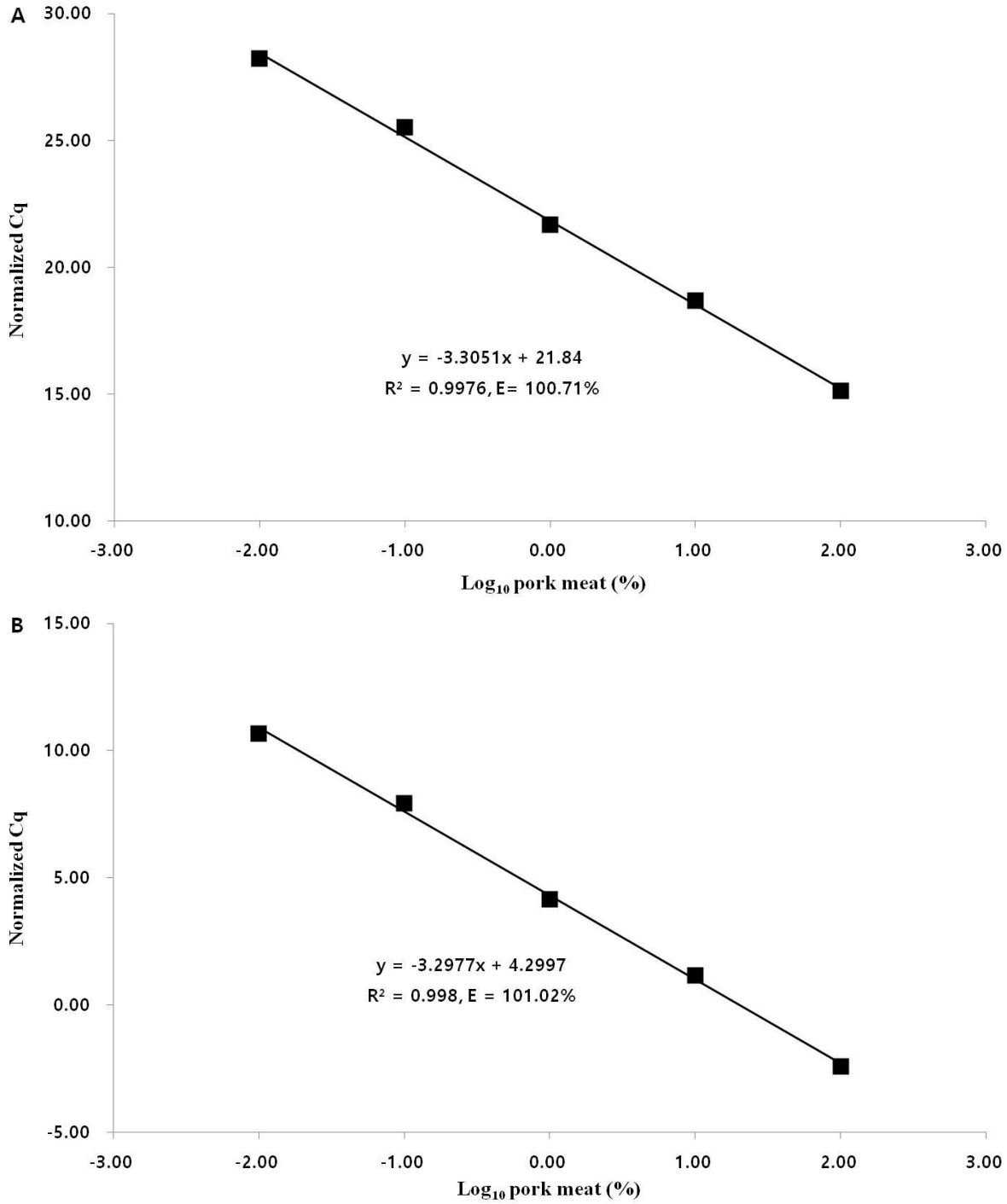


Figure 2.3 Standard curves normalized by Methods #4 (A) and #5 (B).

2.4.5 LOD and LOQ

For the further confidence of the LOD and LOQ of the pork qPCR assay, DNA extracts from the heated-treated binary mixtures (0.1%, 0.01, and 0.001%; w/w), was tested using twenty replicates (Table 2.2). According to the definitions, as described in Materials and Methods, the LOD and LOQ were found to be 0.01% of pork meat, showing positive results in 20 of 20 replicates and RSD of 21.67%. Our result was in well accordance with the qPCR results, as previously reported by Amaral et al. (2017) where LOQ was 0.01% of pork meat in both raw and heat-treated samples.

Table 2.2 Determination of the LOD and LOQ of the qPCR assay.

Pork (%, w/w)	Cq value		Mean		SD (%)[b]	RSD (%)[c]
			Cq value	Estimated pork (%, w/w)[a]		
0.1	25.05	25.28	25.17	0.0980	0.009	9.30
	25.17	25.13				
	25.32	25.03				
	25.16	25.16				
	25.27	25.14				
	25.30	24.91				
	25.42	25.12				
	25.35	25.33				
	25.09	25.08				
	25.16	24.99				
0.01	28.77	28.20	28.40	0.0105	0.002	21.67
	28.71	28.27				
	29.02	28.25				
	28.50	27.92				
	29.24	28.02				
	28.29	28.00				
	28.48	28.28				
	28.53	28.24				
	28.22	28.23				
	28.20	28.70				
0.001	29.29	29.11	29.31	0.0057	0.0016	28.83
	29.16	29.26				
	29.39	N.D.[d]				
	N.D.	29.09				
	28.67	29.35				
	28.81	N.D.				
	28.77	N.D.				
	28.92	N.D.				
	29.13	28.90				
	29.02	29.31				

a. DNA concentration was calculated using the equation ($y = -3.298x + 21.84$) of figure 2.1B.
b. SD: Standard deviation.
c. RSD: Relative standard deviation.
d. To obtain average Cq values, not detected (N.D.) samples were given a Cq= 30.

2.4.6 Validation of the quantification approaches

The five different quantitative approaches were validated on the basis of repeatability and trueness. Accordingly, the standard curve corresponding to each quantification method was used to estimate the quantities of pork meat in the model mixtures (50%, 5%, 2.5%, and 0.25%; w/w) and burger (31.1%; w/w). To assess the inter-day variation, expressed as the RSD of results obtained under repeatability conditions, DNA was independently extracted in triplicate, and the qPCR assays were conducted in triplicate on three different days. The repeatability observed using all quantification methods ranged from 3.93% to 15.34% in all model mixtures and burger, satisfying the acceptable criterion (≤ 25%). The trueness, expressed as bias, of each method was summarized in Table 2.3. The bias observed for Method #1 ranged from -17.71 to -32.76, and the acceptable criterion (≤ ±25%) was not satisfied in 5% and 2.5% model mixtures (-26.82% and -32.76%, respectively) and burger (-28.52%). In the case of Method #2, unsatisfied values (-29.31% and -37.79%) were observed in 5% and 0.25% model mixtures, respectively. Method #3 based on the relative standard curve (Figure 2.1A) outperformed Methods #1 and #2 based on the absolute standard curves (Figures 2.2A–C) in terms of the trueness. This value observed using Method #3 was within ±20% with the exception of 2.5% model mixture (-25.22%), which slightly deviated from the acceptable criterion. In the case of Methods #4 and #5 that were normalized by the reference system, the observed bias satisfied the acceptable criterion in all model mixtures, ranging from -21.40% to 7.51% and from -19.10% to 2.34%, respectively. Moreover, these two methods showed better trueness (-7.81% and -6.73%, respectively) in the model burger than that of Methods #1, #2, and #3 (-28.52%, -11.79%, and -18.64%, respectively). Therefore, our results strongly demonstrated that the two normalized methods were superior in the accuracy of results to Methods #1, #2, and #3. They can successfully be used to estimate the

quantities of pork meat in the range of 100%–0.01% in beef products. These two quantification methods were applied to the further qPCR analysis of processed beef products.

Table 2.3 Quantitative results obtained for model mixtures using the four different quantification methods.

Quantification method	Model mixture[a]	Estimated value (%, w/w)[b]			Mean (%)	SD (%)[c]	RSD (%)[d]	Bias (%)
	(pork %, w/w)	Day 1	Day 2	Day 3				
1	A (50%)	40.85	42.34	40.24	41.14	1.97	4.79	-17.71
	B (5%)	3.79	3.89	3.30	3.66	0.39	10.61	-26.85
	C (2.5%)	1.55	1.70	1.80	1.68	0.14	8.15	-32.76
	D (0.25%)	0.20	0.22	0.19	0.20	0.02	9.04	-18.85
	E (31.1%)	22.88	22.09	21.72	22.23	1.10	4.93	-28.52
2	A (50%)	40.12	47.25	44.96	44.11	3.89	8.82	-11.78
	B (5%)	3.71	3.57	3.33	3.53	0.45	12.83	-29.31
	C (2.5%)	1.99	2.31	2.22	2.17	0.20	9.42	-13.05
	D (0.25%)	0.14	0.16	0.16	0.16	0.02	12.51	-37.79
	E (31.1%)	27.63	27.35	27.34	27.44	1.14	4.14	-11.77
3	A (50%)	46.75	48.47	46.04	47.09	2.27	4.83	-5.83
	B (5%)	4.24	4.36	3.69	4.10	0.44	10.70	-18.08
	C (2.5%)	1.72	1.89	2.00	1.87	0.15	8.22	-25.22
	D (0.25%)	0.22	0.24	0.21	0.22	0.02	9.12	-11.43
	E (31.1%)	26.05	25.14	24.71	25.30	1.43	5.64	-18.64
4	A (50%)	41.47	48.27	46.01	45.25	3.68	8.14	-9.50
	B (5%)	4.23	4.03	3.83	4.03	0.52	13.01	-19.37
	C (2.5%)	2.48	2.87	2.71	2.69	0.25	9.34	7.51
	D (0.25%)	0.17	0.21	0.21	0.20	0.03	15.34	-21.40
	E (31.1%)	28.87	28.58	28.57	28.67	1.13	3.93	-7.81
5	A (50%)	41.53	48.72	46.45	45.57	3.93	8.62	-8.87
	B (5%)	4.25	4.08	3.82	4.05	0.50	12.41	-19.00

C (2.5%)	2.35	2.72	2.60	2.56	0.23	9.09	2.34
D (0.25%)	0.18	0.21	0.21	0.20	0.02	12.14	-19.10
E (31.1%)	29.18	28.92	28.91	29.01	1.15	3.97	-6.73

a. A−E; model mixtures, E; Model burger.
b. Values are the mean of 3 replicates.
c. SD: Standard deviation.
d. RSD: Relative standard deviation.

2.4.7 Estimation of pork quantity in commercial beef products

A total of fourteen commercial beef products were analyzed in order to identify the pork meat adulteration, and their labels only declared information regarding meat species used (e.g., beef), but not the percentage of each meat (Table 2.4). For the quantification of pork meat, DNA was independently extracted from the beef products in duplicate, and the qPCR assays were performed using eight replicates on two different days. The beef-specific qPCR results showed that the average Cq values obtained for all commercial samples ranged from 14 to 17 cycles, among which, in ten samples, pork DNA was not detected using the pork-specific qPCR assay. Thus, the results of the ten samples were consistent with their labeling information. In contrast, pork meat was identified in three samples (Samples #6, #7, and #8), despite the statement on their labels describing beef as the only meat ingredient. However, the amounts estimated for Samples #6, #7, and #8, using Methods #4 and #5, were in the range of 0.01 to 0.03%, which were near to the LOQ. Additionally, the type of these three products was pressed hams that are generally manufactured through several steps, including chopping, pressing, and slicing. When multiple ingredients are ground and mixed on the same equipment without sufficient cleaning, unintentional mislabeling may occur. A recent market survey conducted in Canada used 1% of cut-off value to distinguish the sausage products where undeclared species detection may be due to trace contamination rather than purposeful adulteration (Naaum, et al., 2018). Therefore, when considering this information, the mislabeling found in this study would be possible cross-contamination at the manufacturing facility. One sausage product (Samples #14) declared beef and pork as meat ingredients on its label; however, beef DNA was not detected by the beef-specific qPCR assay. Furthermore, the pork meat amount estimated by the quantification methods was found

to be up to 81.11%, indicating that this mislabeling would be an instance of food fraud for economic gain.

Table 2.4 Results of the application of qualitative and quantitative real-time PCR systems to commercial beef products.

Sample	Product	Species declared on label	Mean Cq value (± SD)[a]			Estimated pork meat content (%, w/w)					
			18S rRNA	Beef	Pork	Method 4			Method 5		
						Mean	SD[b]	RSD[c]	Mean	SD[b]	RSD[c]
S1	Ground beef	Beef	N.T.	16.68±0.12	N.D.	-	-	-	-	-	-
S2	Seasoned roast beef	Beef	N.T.	16.56±0.20	N.D.	-	-	-	-	-	-
S3	Seasoned roast beef	Beef	N.T.	14.61±0.29	N.D.	-	-	-	-	-	-
S4	Seasoned roast beef	Beef	N.T.	15.74±0.33	N.D.	-	-	-	-	-	-
S5	Jerky	Beef	N.T.	14.32±0.12	N.D.	-	-	-	-	-	-
S6	Pressed ham	Beef	17.03±0.15	15.17±0.02	26.27±0.27	0.03	0.01	20.83	0.03	0.01	18.35
S7	Pressed ham	Beef	17.33±0.10	14.41±0.09	27.88±0.29	0.01	0.00	21.00	0.01	0.00	20.29
S8	Pressed ham	Beef	18.40±0.06	14.96±0.01	28.39±0.36	0.03	0.01	26.18	0.02	0.01	26.94
S9	Beef burger	Beef	N.T.	15.71±0.01	N.D.	-	-	-	-	-	-
S10	Meatball	Beef	N.T.	15.43±0.15	N.D.	-	-	-	-	-	-
S11	Meatball	Beef	N.T.	14.77±0.24	N.D.	-	-	-	-	-	-
S12	Sausage	Beef	N.T.	17.71±0.02	N.D.	-	-	-	-	-	-
S13	Sausage	Beef	N.T.	15.36±0.22	N.D.	-	-	-	-	-	-
S14	Sausage	Pork and beef	17.70±0.12	N.D.	15.71±0.19	79.34	6.34	7.99	81.11	6.29	7.76

a. Values are the mean of 8 replicates.
b. SD: Standard deviation.
c. RSD: Relative standard deviation.
N.T.: not tested, N.D.: not detected.

2.5. Discussion

In this study, we assessed the five different quantification methods, which have been extensively used in qPCR assays for meat products, in terms of the precision (RSD) and accuracy (bias) of results. As seen in Figures 2.1–2.3, standard curves for each quantification method showed comparable R^2 and E values (0.988–0.998 and 99.77%–107.11%, respectively). In addition, RSD values observed for the method validation were acceptable and comparable in all quantification methods (3.93%–15.34%). However, each quantification method revealed the significant differences in the accuracy of results. As shown in Table 2.3, accuracy was better in order of the methods from #5 to #1. Therefore, our results clearly indicated that the proper choice in quantification approaches mostly affect the accuracy, and the normalization approaches (e.g., Methods #4 and #5) could significantly improve the trueness. In contrast, these approaches could not demonstrate a significant improvement in the precision of results.

Method #1 has been conveniently used to quantify target species DNA in meat products (Hossain, et al., 2017). Mitochondrial DNA is the most preferred target sequence for meat species identification by qPCR assays; however, the amount of these genes varies in different tissues and species (Ballin, Vogensen, & Karlsson, 2009). This can lead to biased results generated by Method #1, unless sample origin and composition are similar to those of the standard curve. To compensate for this potential inaccuracy, matrix-adapted standards were introduced, and the use of matrix-adapted calibrators showed higher precision and accuracy compared with calibration using DNA dilutions from muscle tissues in qPCR assays for beef, pork, chicken, and turkey in sausages (Eugster, Ruf, Rentsch, Hübner, & Köppel, 2008; Eugster, Ruf, Rentsch, & Köppel, 2009). However, different matrix-adapted reference materials must be prepared for the accurate quantification of each sample type. In this regard,

Method #3 should show better performance than Method #1. In the model burger that had multiple matrices different from the reference binary mixtures, higher bias was observed for Method #3 (-18.64%) compared with Methods #2, #4, and #5 (-11.77, -7.81, and -6.73, respectively) normalized by the reference system. In the quantification of Sample #14, Methods #1 and #3 estimated 63.23% and 72.86% of pork meat, respectively, whereas Methods #2, #4, and #5 estimated more reliable percentages (80.23%, 79.34, and 81.11%, respectively) (Figure 2.4). RSD values for all methods were in the range from 7.76% to 13.18%. Therefore, our findings suggested that the methods normalized by the reference system were more appropriate for the quantification of target species used in processed meat products.

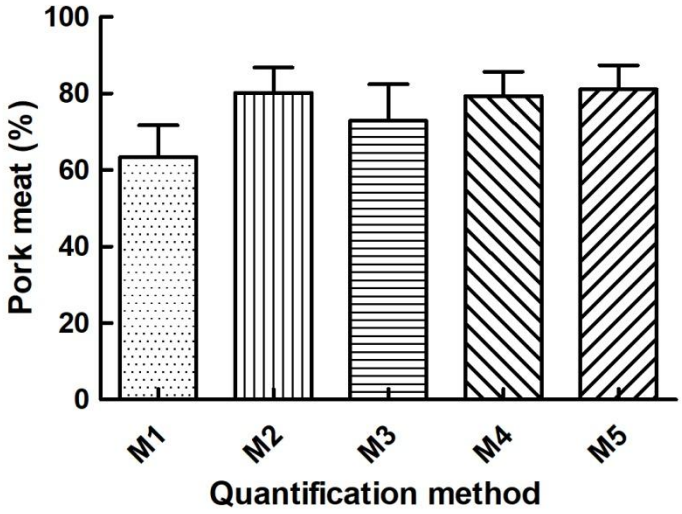


Figure 2.4 Comparison of pork meat quantities in Sample #14 estimated by the five different methods.

So far, three different normalization approaches have been reported for qPCR assays, as described in Materials and Methods, and the accuracy and precision of each method have been well validated in many previous quantification studies (Amaral, Santos, Oliveira, & Mafra, 2017; Druml, Grandits, Mayer, Hochegger, & Cichna-Markl, 2015; Druml, Mayer, Cichna-Markl, & Hochegger, 2015; Fajardo, et al., 2008; Iwobi, et al., 2015; Iwobi, et al., 2017; M. Kim, Yoo, Lee, Hong, & Kim, 2016; Meira, Costa, Villa, Ramos, Oliveira, & Mafra, 2017; Rojas, et al., 2010; Soares, Amaral, Oliveira, & Mafra, 2013). As shown in Table 2, Methods #4 and #5 outperformed Method #2 in our comparative study. It should be noted that Method #2 was originally designed and developed based on single-copy species-specific (i.e., epidermal growth factor pseudogene, lactoferrin, cyclic GMP phosphodiesterase, beta-actin, and growth hormone receptor) and reference (myostatin) genes. This approach has been successfully used to obtain comparable quantitative results from different species (deer, beef, pork and horse); however this approach can reduce the sensitivity of qPCR assays (Druml, Grandits, Mayer, Hochegger, & Cichna-Markl, 2015; Druml, Mayer, Cichna-Markl, & Hochegger, 2015; Iwobi, et al., 2015; Iwobi, et al., 2017; Laube, Zagon, Spiegelberg, Butschke, Kroh, & Broll, 2007). In this study, multi-copy species-specific (*cytb*) and reference (18*S* rRNA) genes were used for the qPCR assays, generally showing improved sensitivity. Thus, the difference in copy numbers of target genes may cause the inaccuracy observed for Method 2 (Table 2.3). Therefore, our findings clearly suggested that the analytical target, type, and sensitivity should be considered, by which adequate species-specific and reference systems are carefully adapted for obtaining accurate quantitative results.

2.6 Conclusions

Although qPCR methods can reportedly quantify the amounts of target species used in meat products, this quantification is still challenging. Thus, in this study, with the efforts to improve the precision and accuracy of qPCR results, we compared and assessed the five different quantification methods for the first time. Our findings clearly indicated that 1) the spectrofluorometric DNA quantification method is more appropriate for qPCR assays requiring high sensitivity for processed food products, 2) a suitable reference system that shows the amplification efficiency comparable to that of a species-specific system should be carefully selected according to the analytical target and type, 3) qPCR results normalized by the reference system should improve the accuracy of quantification for processed meat products.

Chapter 3[iii]

A rapid real-time PCR method to differentiate between mottled skate (*Beringraja pulchra*) and other skate and ray species

[iii] This chapter has been published: Mi-Ra Kim, Kisung Kwon, Yoo-Kyung Jung, Tae Sun Kang. 2018. A rapid real-time PCR method to differentiate between mottled skate (*Beringraja pulchra*) and other skate and ray species. Food Chemistry, 255:112-119. This chapter is reproduced in this thesis with the permission of Elsevier.

3.1 Abstract

Skates and rays are commercially important fish in South Korea, and among them, *Beringraja pulchra* has the highest economic value. However, the similar morphological traits among skates and rays are often exploited for seafood fraud. Here, we designed both *Beringraja pulchra*-specific and skate-universal primer sets, capable of detecting short sequences in the cytochrome oxidase subunit I gene, and developed highly sensitive and reliable quantitative real-time PCR (qPCR) assays to differentiate between *Beringraja pulchra* and other skate and ray species. A ΔCq method based on differences in the amplification efficiency was developed, validated, and then used to confirm the presence of *Beringraja pulchra* in twenty-six commercial skate products. The average ΔCq value obtained for other skate species (18.94 ± 3.46) was significantly higher than that of *Beringraja pulchra* (1.18 ± 0.15). For on-site applications, we developed an ultra-fast qPCR assay, allowing for completion of the entire analytical procedure within 30 min.

Keywords: Genetic identification, Seafood authentication, Ultra-fast qPCR, Cytochrome oxidase subunit I, Amplification efficiency

3.2 Introduction

Skates and rays, which are commercially important in South Korea, belong to the Batoidea superorder, which contains more than 500 species in thirteen families (Lago, Vieites, & Espineira, 2012). Among these families, the *Rajidae* is one of the most diverse, with 227 species in twenty-five genera; eleven skate species, belonging to four genera, have been identified in South Korea (Kang, Park, & Jo, 2012). Mottled skate (*Beringraja pulchra*) is the most commercially valuable fish among these species, and can command prices reaching from US$ 10 to 30 per kilogram in South Korea (IUCN, 2017). *Beringraja pulchra* has been found in the Yellow Sea, the Pacific coast of Japan, the East China Sea, the southern Kurils, and even as far as the coast of western Sakhalin (Jeong, Kim, Kim, Myoung, & Lee, 2014; Kang, Park, & Jo, 2012). In South Korea, the average annual catch of this species sharply declined from 2,700 metric tons in the 1990s to 220 metric tons in the 2000s, representing a 90% decrease over a decade. As a result, *Beringraja pulchra* is currently listed as a vulnerable species by the International Union for Conservation of Nature (IUCN, 2017).

The major morphological characteristics of *Beringraja pulchra*, including flattened bodies, enlarged pectoral fins fused to the head, and gill slits placed on the ventral surfaces, are very similar to those of its relatives, namely other skates and rays. In South Korea, *Beringraja pulchra* is generally consumed in fermented, steamed, and/or seasoned forms and therefore it is impossible to identify its presence in processed products. The habitat, reproduction, feeding habits, spawning, and fishing characteristics of *Beringraja pulchra* have been widely studied. However, despite the increase in its value as a food, rapid and efficient *Beringraja pulchra* identification methods have yet to be developed.

The development of a processed-food market, due to improvements in food-processing technologies, has led to an increase in economically motivated adulteration (EMA)

of food, such as the substitution of a valuable authentic constituent with a less expensive ingredient, or the false declaration of either the raw materials' origin, or the production process used to manufacture an ingredient. Generally, the authentication of fish products depends on different morphological or meristic traits. However, in the case of commercially-processed foods, it is impossible to authenticate the original species by morphological characteristics alone. Therefore, considerable research efforts, focusing on product authentication, have been conducted with the aim of developing accurate identification methods for the species present in various processed products. Several methods have been developed for the identification of fish and seafood products, including isoelectric focusing (IEF), capillary electrophoresis (CE), high-performance liquid chromatography (HPLC), and immunoassays (Rasmussen & Morrissey, 2008). However, these protein-based methods have two major drawbacks affecting the quality of analysis: protein denaturation during manufacturing and insufficient resolution power for differentiating between closely-related species. Therefore, DNA-based methods are considered a better alternative due to the high specificity of DNA and to its greater stability in highly-processed food. Various polymerase chain reaction (PCR)-based techniques have been widely used for the identification of commercially important seafood species, such as DNA barcoding (Hellberg & Morrissey, 2011; Rasmussen & Morrissey, 2008), forensically informative nucleotide sequencing (FINS) (Bartlett & Davidson, 1992), microsatellite analysis (Kang, Park, & Jo, 2012), PCR-restriction fragment length polymorphism (PCR-RFLP) analysis (Cho, et al., 2014), and PCR with specific primers (Lee, Kim, Jo, Jung, Kwon, & Kang, 2016; Wen, Hu, Zhang, & Fan, 2012). Quantitative real-time PCR (qPCR), which allows the detection and quantification of PCR products formed during the amplification process without post-PCR steps, is an emerging technique that is increasingly being used for the identification of fish species. In fact, qPCR systems have been used to identify different fish species such as mackerel

(Velasco, Sanchez, Martinez, Santaclara, Perez-Martin, & Sotelo, 2013), cod (Herrero, Madrinan, Vieites, & Espineira, 2010), hake (Sanchez, Quinteiro, Rey-Mendez, Perez-Martin, & Sotelo, 2009), skate (Hwang, Lee, Kim, Jo, Choi, Kang, et al., 2015), and ling (Taboada, Sanchez, & Sotelo, 2017).

In this study, we aimed to develop an assay that could authenticate and differentiate between the mottled skate and other skate and ray species. To achieve this, we developed a highly-accurate qPCR assay using both *Beringraja pulchra*-specific and skate-universal primer sets. The system was validated by analyzing cytochrome oxidase subunit I (COI) sequences from twenty-six commercial skate samples. Additionally, we aimed to develop an ultra-fast method for field identification of samples.

3.3 Materials and methods

3.3.1 Samples and DNA extraction

Fresh muscle tissues identified as skate (*Beringraja pulchra* and *Okamejei kenojei*) and ray (*Urolophus aurantiacus* and *Dasyatis akajei*) were obtained from Pukyong National University (Busan, South Korea) and the National Institute of Biological Resources (Incheon, South Korea), and these samples were used as positive controls for the optimization of qPCR conditions. For the validation of the qPCR assays, a total of twenty-six different commercially available samples, described as fermented skate products, were purchased from markets in the Jeolla province of South Korea and via the Internet (Table 3.1).

For DNA extraction from fermented products containing seasonings and spices, the samples were washed well with distilled water and preserved in 100% ethanol. DNA was extracted from 30 mg of muscle tissue selected from each sample using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Briefly, the samples were mixed with 180 μL tissue lysis buffer (ATL) and 20 μL protease K, and the mixture was incubated at 56℃ until complete lysis. The lysis solution was then mixed with 200 μL lysis buffer (AL) and 200 μL ethanol (96% to 100%), and the lysate was transferred to a DNeasy Mini spin column. After centrifugation at 6000 × *g* for 1 min, the column was washed twice with 500 μL washing buffer (AW1 and AW2), and purified DNA was eluted by adding 100 μL elution buffer (AE).

The extracted total DNA was quantified using a NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Delaware, USA). DNA concentrations were determined by measuring UV absorbance at 260 nm (1 absorbance unit corresponds to 50 μg/mL double-stranded DNA). The purity of the extract was determined by calculating the ratio of absorbance measured at 260 nm to that measured at 280 nm.

Table 3.1 Reference and commercial samples used in this study.

Samples	Products	Origin of Skate[1]	FINS result			qPCR result (average Cq value)			Ultra-fast qPCR result
			Accession No.	Identity (%)	Species	*Beringraja pulchra*	Skate universal	ΔCq[2]	ΔCq[2]
1	Fresh	PNU and NIBR	KR676447.1	100	*Beringraja pulchra*	15.09 ± 0.26	13.56 ± 0.12	1.53 ± 0.38	1.93 ± 0.05
2	Fresh	PNU and NIBR	NC007173.1	100	*Okamejei kenojei*	34.71 ± 0.62	13.77 ± 0.09	20.94 ± 0.63	20.36 ± 0.08
3	fresh	PNU and NIBR	EU339355.1	100	*Urolophus aurantiacus*	35.15 ± 1.08	34.77 ± 0.78	-	-
4	Fresh	PNU and NIBR	EU339356.1	100	*Dasyatis akajei*	36.14 ± 1.66	38.77 ± 0.52	-	-
5	Fermented	Argentina	EU074400.1	100	*Dipturus chilensis*	37.90 ± 0.10	17.98 ± 0.01	19.93 ± 0.01	17.76 ± 0.11
6	Fermented	Unidentified	EU074400.1	100	*Dipturus chilensis*	N.D.	28.44 ± 0.01	11.57 ± 0.01	17.92 ± 0.11
7	Fermented	Argentina	EU074400.1	100	*Dipturus chilensis*	38.51 ± 0.01	20.39 ± 0.04	18.13 ± 0.04	17.76 ± 0.10
8	Fermented	Unidentified	JF895057.1	100	*Dipturus laevis*	N.D.	15.46 ± 0.28	24.55 ± 0.28	20.92 ± 0.62
9	Seasoned	Argentina	EU074400.1	100	*Dipturus chilensis*	N.D.	21.10 ± 0.31	18.90 ± 0.31	16.80 ± 0.08
10	Seasoned	Chile	EU074400.1	100	*Dipturus chilensis*	N.D.	18.03 ± 0.15	21.98 ± 0.15	19.15 ± 0.71
11	Seasoned	Argentina	EU074404.1	100	*Dipturus chilensis*	38.80 ± 0.03	18.80 ± 0.04	20.00 ± 0.05	15.32 ± 0.71
12	Seasoned	Argentina	EU074400.1	100	*Dipturus chilensis*	38.94 ± 0.01	19.41 ± 0.02	19.54 ± 0.03	17.33 ± 0.72
13	Fermented	Unidentified	EU074404.1	100	*Dipturus chilensis*	34.04 ± 0.01	19.14 ± 0.23	14.90 ± 0.23	16.24 ± 0.78
14	Fermented	Argentina	KF648508.1	100	*Dipturus chilensis*	37.03 ± 0.04	19.17 ± 0.73	17.86 ± 0.73	19.26 ± 0.31
15	Fermented	South Korea	KR676447.1	100	*Beringraja pulchra*	17.84 ± 0.21	16.82 ± 0.01	1.02 ± 0.20	2.02 ± 0.99
16	Fermented	South Korea	KR676447.1	100	*Beringraja pulchra*	16.31 ± 0.07	15.20 ± 0.11	1.12 ± 0.13	2.86 ± 0.20
17	Fermented	South Korea	KR676448.1	100	*Beringraja pulchra*	19.21 ± 0.03	18.10 ± 0.04	1.11 ± 0.05	1.85 ± 0.21
18	Fermented	South Korea	KR676448.1	100	*Beringraja pulchra*	16.29 ± 0.04	15.14 ± 0.01	1.15 ± 0.04	1.69 ± 0.04
19	Fermented	South Korea	KR676447.1	99	*Beringraja pulchra*	15.80 ± 0.04	14.77 ± 0.01	1.03 ± 0.04	0.91 ± 0.04
20	Fermented	South Korea	KR676448.1	100	*Beringraja pulchra*	15.84 ± 0.08	14.69 ± 0.01	1.15 ± 0.08	2.00 ± 0.08
21	Fermented	South Korea	KR676447.1	100	*Beringraja pulchra*	18.08 ± 0.06	16.78 ± 0.08	1.30 ± 0.10	1.42 ± 0.58
22	Fermented	South Korea	KR676448.1	100	*Beringraja pulchra*	14.59 ± 0.05	13.61 ± 0.10	0.98 ± 0.11	1.81 ± 0.90
23	Fermented	South Korea	KR676447.1	99	*Beringraja pulchra*	14.88 ± 0.01	13.68 ± 0.05	1.20 ± 0.05	2.31 ± 0.49
24	Fermented	South Korea	KR676447.1	100	*Beringraja pulchra*	13.69 ± 0.01	12.24 ± 0.13	1.45 ± 0.13	2.68 ± 0.47
25	Fermented	South Korea	KR676448.1	100	*Beringraja pulchra*	16.70 ± 0.02	15.50 ± 0.01	1.20 ± 0.02	2.38 ± 0.56
26	Fermented	South Korea	KR676448.1	100	*Beringraja pulchra*	16.07 ± 0.01	14.88 ± 0.00	1.19 ± 0.01	2.85 ± 0.11

27	Fermented	South Korea	KR676448.1	100	*Beringraja pulchra*	16.41 ± 0.08	15.27 ± 0.04	1.14 ± 0.09	1.54 ± 0.71
28	Fermented	South Korea	KR676448.1	100	*Beringraja pulchra*	16.88 ± 0.00	15.59 ± 0.06	1.29 ± 0.06	1.16 ± 0.23
29	Fermented	South Korea	KR676448.1	100	*Beringraja pulchra*	17.71 ± 0.08	16.57 ± 0.03	1.14 ± 0.08	2.13 ± 0.58
30	Fermented	South Korea	KR676448.1	100	*Beringraja pulchra*	15.19 ± 0.04	14.18 ± 0.05	1.01 ± 0.06	2.81 ± 0.02

1. PNU and NIBR represent Pukyong National University and the National Institute of Biological Resources, respectively, and origin information was declared in the labels of commercial products.
2. ΔCq = average Cq value of *B. pulchra* specific qPCR - average Cq value of skate universal qPCR. To obtain average ΔCq values, not detected (N.D.) samples were given a Cq= 40.

3.3.2 Target gene selection and oligonucleotide primers

COI gene sequences from different skate and ray species were downloaded from the National Center for Biotechnology Information (NCBI), and a multiple sequence alignment was constructed using these sequences using the BioEdit software, version 7.2.2. Species-specific primers used in this study targeted *Beringraja pulchra* (accession No. KR676448.1), *O. kenojei* (accession No. NC007173.1), *U. aurantiacus* (accession No. EU339354.1), and *Dasyatis akajei* (accession No. NC021132.1) mitochondrial COI genes (Figure 3.1A and Table 3.2). For the identification of skate species, a universal primer set was designed based on COI gene sequences from *Beringraja pulchra* (accession No. KR676448.1), *O. kenojei* (accession No. NC007173.1), *Okamejei acutispina* (accession No. EU334812.1), *Raja koreana* (accession No. EU339351.1), and *Dipturus kwangtungensis* (accession No. EU339347.1), against the ray species genes, *Dasyatis matsubaras* (accession No. EU339363.1), *Platyrhina sinensis* (accession No. HM180795.1), *Bathyraja sinoterus* (accession No. FJ869229.1), and *Bathyraja violacea* (accession No. FJ164396.1) (Figure 3.1B and Table 3.2). The theoretical specificity of the primer sets was checked using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Ye, Coulouris, Zaretskaya, Cutcutache, Rozen, & Madden, 2012) and the GenBank database.

A

```
KR676448.1    CCAGTCCTAG CAGCCGGCAT CACTATACTG CTCACAGATC GTAATCTCAA
NC_007173.1   CCAGTTCTAG CAGCTGGCAT CACCATACTT CTTACAGATC GTAATCTCAA
NC_021132.1   CCAGTTCTAG CAGCGGGCAT TACTATGCTT CTCACAGATC GAAATCTCAA
EU339354.1    CCTGTTTTAG CAGCAGGCAT CACCATACTT CTTACAGATC GCAACCTCAA

KR676448.1    CACAACTTTC TTTGACCCGG CAGGAGGAGG GGACCCCATC CTATACCAAC
NC_007173.1   CACAACTTTC TTTGACCCGG CGGGAGGAGG CGACCCAACT CTATACCAGC
NC_021132.1   CACAACTTTC TTTGATCCAG CAGGTGGAGG AGACCCTATT CTCTATCAAC
EU339354.1    TACAACCTTC TTTGACCCCG CAGGAGGGGG GGACCCCATT CTCTATCAAC

KR676448.1    ACTTATTCTG ATTCTTCGGC CATCCTGAGG TCTACATTTT GATTCTACCT
NC_007173.1   ACTTATTCTG ATTCTTCGGA CACCCAGAAG TCTACATCCT AATTCTTCCA
NC_021132.1   ATCTCTTCTG ATTCTTTGGA CACCCAGAAG TTTATATTCT TATTCTACCA
EU339354.1    ACCTC----- ---------- ---------- ---------- ----------
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B

```
KR676448.1    CATTTAGCAG GTGTTTCATC TATCCTGGCC TCCATTAACT TCATCACCAC
NC_007173.1   CACTTGGCAG GCATCTCATC TATCCTAGCC TCCATTAACT TCATCACCAC
EU334812.1    CACTTGGCAG GCATCTCATC TATCCTCGCC TCCATTAACT TCATCACCAC
EU339351.1    CACTTAGCAG GTATTTCATC TATTCTGGCC TCCATTAACT TCATCACCAC
EU339347.1    CACTTGGCAG GTATTTCATC TATTCTGGCC TCCATTAACT TCATCACCAC
EU339363.1    CATTTAGCCG GTGTTTCCTC TATCCTAGCA TCCATTAACT TTATTACAAC
HM180795.1    CACTTAGCTG GAGTTTCCTC CATTCTAGCC TCAATTAATT TTATTACTAC
FJ869229.1    CACTTAGCCG GAATCTCATC TATCTTAGCA TCAATTAATT TCATTACTAC
FJ164396.1    CACTTAGCCG GAATCTCATC TATCTTAGCA TCAATTAATT TTATTACTAC

KR676448.1    AATTATCAAC ATAAAACCAC CAGCAATCTC TCAATACCAA ACACCTTTAT
NC_007173.1   AATTATCAAT ATAAAACCAC CAGCAATCTC CCAATACCAA ACACCATTAT
EU334812.1    AATTATTAAT ATAAAACCAC CAGCAATCTC TCAGTACCAA ACACCATTAT
EU339351.1    AATTATTAAC ATGAAGCCAC CAGCAATCTC TCAATACCAA ACACCCTTAT
EU339347.1    AATTATTAAC ATAAAACCAC CGGCAATCTC TCAGTACCAA ACACCCTTAT
EU339363.1    AATTATTAAC ATAAAACCCC CTGCAATTTC CCAATACCAA ACACCTCTCT
HM180795.1    TATCATCAAT ATAAAACCAC CAATAATTTC TCAATATCAG ACATCCCTTT
FJ869229.1    TATTATTAAT ATAAAGCCAC CAGCAATTTC ACAATACCAA ACACCTTTAT
FJ164396.1    TATTATTAAT ATAAAACCAC CAGCAATTTC ACAATACCAA ACACCTTTAT

KR676448.1    TCGTATGATC AATTCTTGTT ACAACTGTCT TGCTTCTTAT GGCCCTCCCA
NC_007173.1   TCGTATGATC AATTCTTGTT ACAACTGTCT TACTTCTTAT AGCCCTACCA
EU334812.1    TCGTATGATC AATTCTTGTT ACAACTGTCT TACTTCTTAT AGCCCTGCCA
EU339351.1    TCGTCTGATC AATTCTTGTT ACAACCGTCT TACTTCTTAT AGCCCTCCCA
EU339347.1    TCGTGTGATC AATTCTTGTT ACAACTGTCT TACTTCTTAT AGCCCTCCCA
EU339363.1    TTGTTTGATC CATCCTCATT ACAACAATCC TCCTTTTACT ATCACTCCCA
HM180795.1    TTGTTTGATC TATTCTTGTA ACAACCGTTC TCCTACTCCT CTCGTTACCT
FJ869229.1    TTGTCTGATC AGTTCTTGTT ACAACTGTAC TACTTCTTTT AGCTCTTCCA
FJ164396.1    TTGTATGATC AGTTCTTGTC ACAACTGTAC TACTTCTTTT AGCTCTCCCA

KR676448.1    GTCCTAGCAG CCGGCATCAC TATACTGCTC ACAGATCGTA ATCTCAACAC
NC_007173.1   GTTCTAGCAG CTGGCATCAC CATACTTCTT ACAGATCGTA ATCTCAACAC
EU334812.1    GTACTAGCAG CTGGCATCAC CATACTTCTT ACGGATCGTA ATCTCAATAC
EU339351.1    GTTCTAGCAG CCGGCATCAC TATACTTCTT ACAGATCGTA ATCTCAACAC
EU339347.1    GTTCTAGCAG CCGGCATCAC TATATTACTC ACGGATCGTA ATCTCAACAC
EU339363.1    GTTCTAGCAG CGGGCATTAC TATACTTCTT ACAGACCGTA ATCTTAACAC
HM180795.1    GTATTAGCAG CTGGCATTAC TATACTTCTT ACGGATCGTA ACCTAAACAC
FJ869229.1    GTTTTAGCAG CAGCTATTAC TATACTTTTA ACAGATCGTA ATCTTAACAC
FJ164396.1    GTTTTAGCAG CAGCTATTAC TATACTTTTA ACAGATCGTA ATCTTAACAC

KR676448.1    AACTTTCTTT GACCCGGCAG GAGGAGGGGA CCCCATCCTA TACCAACACT
NC_007173.1   AACTTTCTTT GACCCGGCGG GAGGAGGCGA CCCAACTCTA TACCAGCACT
EU334812.1    AACTTTCTTT GACCCAGCGG GAGGGGGCGA CCCAATTCTA TATCAACACT
EU339351.1    AACTTTCTTT GATCCAGCTG GAGGAGGTGA CCCCATTCTG TACCAACACC
EU339347.1    AACTTTCTTT GACCCGGCAG GGGGAGGGGA CCCCATTCTA TACCAACACT
EU339363.1    AACCTTCTTC GACCCAGCAG GTGGAGGAGA CCCCATTCTC TATCAACATC
HM180795.1    AACCTTCTTT GACCCTGCAG GAGGGGGGGA CCCAATCTTA TACCAACACC
FJ869229.1    AACTTTCTTC GA-------- ---------- ---------- ----------
FJ164396.1    AACTTTCTTC GATCCTGCAG GAGGAGGAGA TCCCATTTTA TATCAACA--
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Figure 3.1 (A) Alignment of COI gene sequences of *Beringraja pulchra* (accession No. KR676448.1), *Okamejei kenojei* (accession No. NC007173.1), *Urolophus aurantiacus* (accession No. EU339354.1), and *Dasyatis akajei* (accession No. NC021132.1), and *Beringraja pulchra*-specific primer-binding sites (yellow sequences). (B) Alignment of COI gene sequences of *Beringraja pulchra* (accession No. KR676448.1), *Okamejei kenojei* (accession No. NC007173.1), *Okamejei acutispina* (accession No. EU334812.1), *Raja koreana* (accession No. EU339351.1), *Dipturus kwangtungensis* (accession No. EU339347.1), *Dasyatis matsubaras* (accession No. EU339363.1), *Platyrhina sinensis* (accession No. HM180795.1), *Bathyraja sinoterus* (accession No. FJ869229.1), and *Bathyraja violacea* (accession No. FJ164396.1), with the skate-universal primer-binding sites shown as yellow sequences.

Table 3.2 Primers used in this study.

Primer	Target gene	Sequence (5'→3')	Size (bp)	Source
rajaF	COI	CCGGCATCACTATACTGCTCA	131	This study
rajaR		AATCAAAATGTAGACCTCAGGATGG		
skateF	COI	CTCCATTAACTTCATCACCACAATT	179	This study
skateR		AGAAAGTTGTGTTGAGATTACGATC		
FISHF2	COI	TCGACTAATCATAAAGATATCGGCAC	655	Ward et al. (2005)
FISHR1		TAGACTTCTGGGTGGCCAAAGAATCA		

3.3.3 qPCR conditions and DNA sequencing

qPCR analysis was performed in a total volume of 20 µL, containing 10 ng of template DNA, 0.5 µM of each primer, 10 µL of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California, USA), and sterile distilled water. The reactions were performed using QuantStudio 3 Real-Time PCR System (Applied Biosystems) under the conditions described in Table 3.3. A non-template control was used as the negative control for the PCR assay, and the PCR specificity and product detection were verified by examining the temperature-dependent melting curves of the PCR products, which were further analyzed using a QIAxcel Advanced system with 100 bp to 2.5 kb size markers and 15 bp to 3.0 kb alignment markers (Qiagen). In order to verify the sequences of the short-length fragments produced by the species-specific and universal primers, the PCR products were eluted from agarose gels and cloned into the pGEMT-easy vector (Promega, Madison, USA). Plasmid DNA was purified using the AccuPrep PCR Purification kit (Bioneer, Seoul, South Korea), and the samples were sent to Bioneer Corp. (Seoul, South Korea) for nucleotide sequence determination.

Table 3.3 Optimized qPCR conditions for skate species analyzed in this study.

Program step	**qPCR**		**Ultra-fast qPCR**	
Hold stage	50 ℃ 95 ℃	(2 min) (10 min)	95 ℃	(30 s)
Amplification stage	95 ℃ 60 ℃	(15 s) (1 min)	95 ℃ 60 ℃ 72 ℃	(5 s) (6 s) (5 s)
Cycle number	40		30	
Melt curve stage (Increment, 0.5℃)	95 ℃ 60 ℃ 95 ℃	(15 s) (1 min) (1 s)	95 ℃ 60 ℃ 95 ℃	(5 s) (70 s)

3.3.4 Acceptable qPCR parameters

Two guidelines were used to define an acceptable qPCR assay (Bustin, et al., 2009). The linear dynamic range should ideally extend over five $\log_{10}$ concentrations, the coefficient of determination (R^2) should be over 0.98, and the amplification efficiency should be in the range of 110% to 90%, corresponding to a slope between -3.1 to -3.6. The limit of detection (LOD) was defined as the lowest concentration at which 95% of the positive samples were still detected. Each quantification cycle (Cq) value was obtained from the average of three replicates. The average Cq value obtained by analyzing other skate and ray species was compared to that of *Beringraja pulchra* samples, using a *t*-test assuming a normal distribution and different variances (Yuan, Reed, Chen, & Stewart, 2006).

3.4.5 FINS identification of commercial skate products

All commercial samples were authenticated using FINS to verify the reliability of qPCR analysis and DNA extraction. DNA from the commercial samples was extracted as described in Section 3.3.1, and the approximately 655-bp large fragments derived from the COI genes were amplified using the primers described previously by Ward et al. (2005) (Table 3.3). Conventional PCR analysis was performed in a total volume of 20 µL, with 10 ng of template DNA, 0.5 µM of each primer, 1 × PCR Buffer, 0.2 mM deoxynucleoside triphosphates (dNTPs), 2.0 mM $MgCl_2$, 1 U rTaq polymerase (TaKaRa Bio Inc., Otsu, Japan), and sterile distilled water. The reactions were performed in a thermal cycler C1000 Touch (Bio-Rad Laboratories, Hercules, California, USA) under the following conditions: 95℃ for 3 min followed by 35 cycles at 94℃ for 30 s, 52℃ for 40 s, and 72℃ for 1 min, and a final extension at 72℃ for 5 min. The sizes and sequences of the PCR amplicons were analyzed as described in Section 3.3.3. Nucleotide sequences were analyzed using BioEdit software and

the data obtained were confirmed using the Basic Local Alignment Search Tool (BLAST) in the NCBI database.

3.3.6 Ultra-fast qPCR conditions

For fast DNA isolation, 50 mg of muscle tissue from each sample was mixed with 500 μL of direct lysis buffer (Rapi:Prep; Genesystem, Daejeon, South Korea), and the mixture was incubated at 25℃ for 5 min with vortexing in 1-min intervals. After brief centrifugation, 10 μL of the lysate was transferred to fresh 1.5-mL micro-tubes containing 90 μL of sterile distilled water, and these samples were used as a template DNA for the ultra-fast qPCR reactions. These PCR reactions were performed in a total volume of 10 μL in a Rapi:Chip (Genesystem) containing 2 μL of template DNA, 0.2 μM of each *Beringraja pulchra*-specific primer (or 0.45 μM of each skate-universal primer), 5 μL of Rapid PCR Master Mix (Genesystem), and sterile distilled water. The ultra-fast reaction was performed using the GENECHECKER Ultra-Fast Real-Time PCR system (Genesystem) under the conditions described in Table 3.3

3.4 Results and Discussion

3.4.1 qPCR assay design

Differentiating between *Beringraja pulchra* and other skate and ray species is challenging because they are closely-related, and therefore, it is important to develop a rapid method with good specificity and sensitivity for the identification of the mottled skate in fresh, fermented, and highly-processed products. To develop a PCR-based method, it should be noted that the target genes are present in multiple copies since the degraded DNA is extracted from highly-processed food samples, and the sequences of target genes should contain the highest interspecific differences combined with the lowest intraspecific variability.

Mitochondrial genes, such as 16S rRNA, cytochrome b, and cytochrome oxidase subunit I and II genes, are widely used for species identification since they are present in high copy number, compared with nuclear DNA copy number, and are highly conserved, enabling the design of specific and sensitive primers (Bartlett & Davidson, 1991). Due to these characteristics, the COI gene has been widely used for marine species identification by PCR-based methods, including for hake, cod, and skate (Herrero, Madrinan, Vieites, & Espineira, 2010; Hwang, et al., 2015; Sanchez, Quinteiro, Rey-Mendez, Perez-Martin, & Sotelo, 2009). Therefore, in this study, primers were designed to detect short sequences within the COI genes, which helps to increase the sensitivity of PCR assays that use fragmented DNA obtained from processed products. *Beringraja pulchra*-specific and skate-universal primer sets were designed to amplify 131-bp and 179-bp long fragments, allowing for the detection of the mottled skate and other skate species. The theoretical specificity of the primer sets was checked using Primer-BLAST against the GenBank database, demonstrating that *Beringraja pulchra*-specific primers are only specific for this species, whereas skate-universal primers can detect other skate species, including *Dipturus nidarosiensis*, *Dipturus laevis*, *Hongeo*

koreana, Okamejei boesemani, *Dipturus springeri*, *Dipturus canutus*, *Dipturus gudgeri*, *Dipturus whitleyi*, *Raja porosa*, and *Dipturus argentinensis*, in addition to the five species (*O. kenojei, Beringraja pulchra, Dipturus kwangtungensis, R. koreana*, and *O. acutispina*) that were used for design of the universal primers.

3.4.2 Specificity and sensitivity of Beringraja pulchra-specific qPCR

Following optimization of the method, the efficiency, sensitivity, specificity, and cross-reactivity of the method were analyzed. A standard curve for the *Beringraja pulchra*-specific qPCR assay was plotted using Cq values and different DNA quantities (10-fold serial dilutions from 10 to 0.001 ng/μL), which yielded a 0.999 linear correlation (R^2) and a slope of -3.309. The amplification efficiency, calculated using the equation: $E = [(10^{(-1/\text{-slope})}\text{-}1] \times 100$, was 100.5%. The LOD for the *Beringraja pulchra* species was 0.001 ng/μL, since all replicates in the linear dynamic range were amplified under 30-cycle conditions (Figure 3.2A). Following qPCR, the size and specificity of the PCR product were analyzed using the QIAxcel Advanced system (131 bp). Temperature-dependent melting curves (Tm: 78.97℃) were defined to confirm the generation of non-specific signals (Figure 3.2A). To confirm the identity and origin of the 131-bp fragment further, the PCR product was eluted from the agarose gel, cloned into the pGEMT-easy vector, and sequenced using M13 sequencing primers. The NCBI BLAST database was screened with the sequences of the PCR product as a query using BLASTn, with the result that the PCR product showed 100% sequence identity to the COI gene of *Beringraja pulchra* (KR676448.1).

The specificity and cross-reactivity of the *Beringraja pulchra*-specific qPCR assay were tested using three species, *O. kenojei*, *U. aurantiacus*, and *Dasyatis akajei*, which are commonly consumed in South Korea. As shown in Figure 3.2B, the average Cq value obtained for the mottled skate DNA samples was 15.09 ± 0.26, whereas no amplification and

false positive results were observed for other species under the stringent 30-cycle conditions. Although a slight signal was observed when *O. kenojei* (34.71 ± 0.62), *U. aurantiacus* (35.15 ± 1.08), and *Dasyatis akajei* (36.14 ± 1.66) samples were analyzed, these Cq values were significantly higher than that obtained for the *Beringraja pulchra* samples, suggesting there was no cross-reaction of the assay with other species.

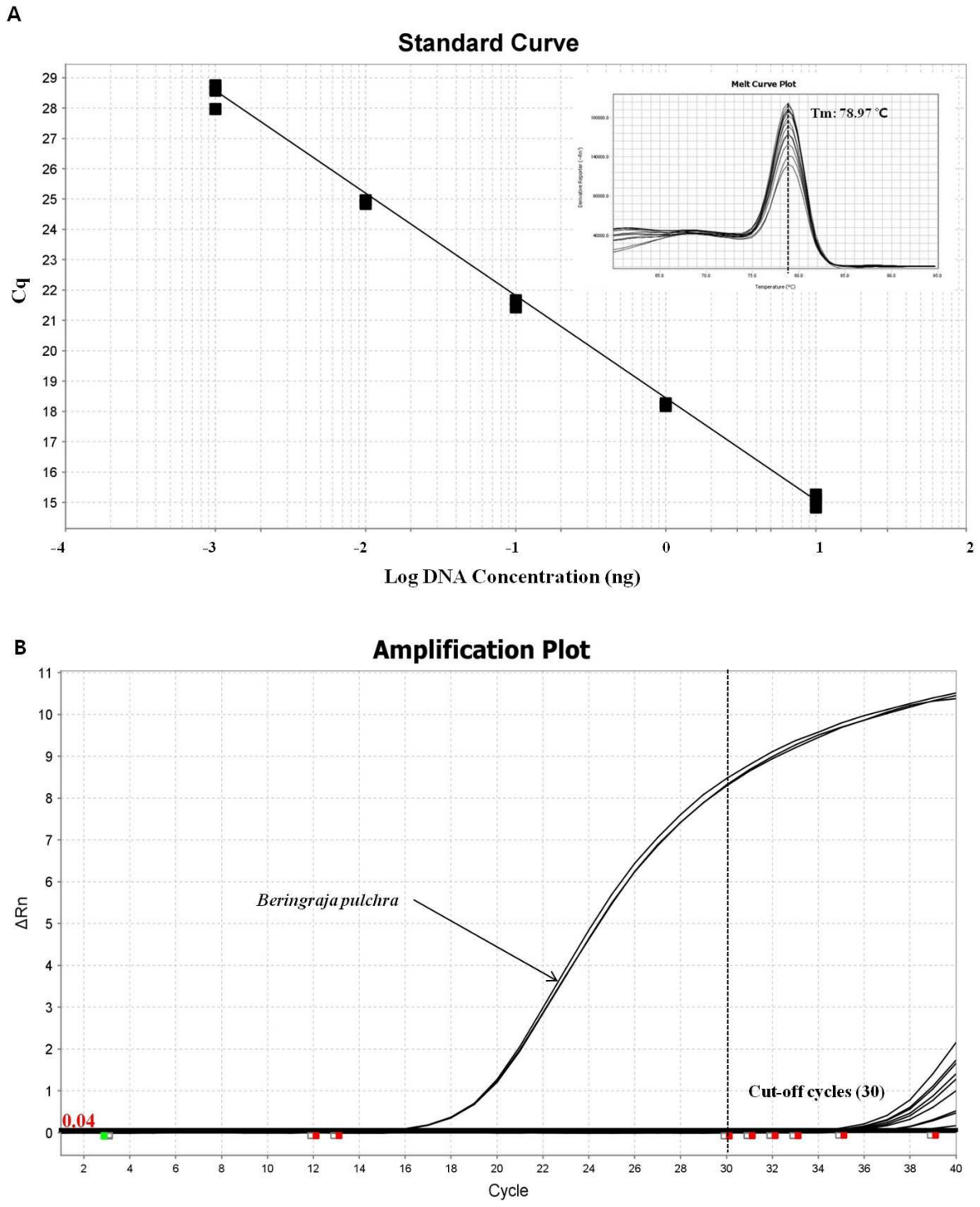


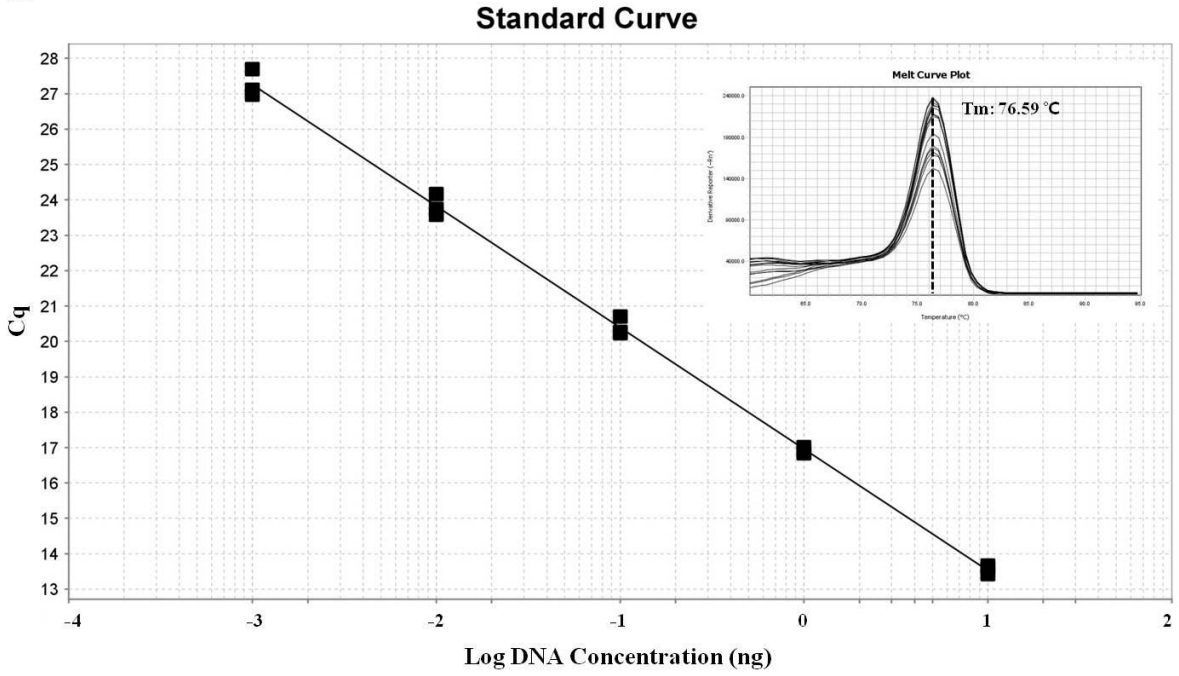
Figure 3.2 (A) Standard curves and melting curves for the *Beringraja pulchra-specific* qPCR assay. (B) Amplification pattern for *Beringraja pulchra* (Cq: 15.09 ± 0.26) and other species (Cq: 35.17 ± 1.32).

3.4.3 Specificity and sensitivity of skate-universal qPCR

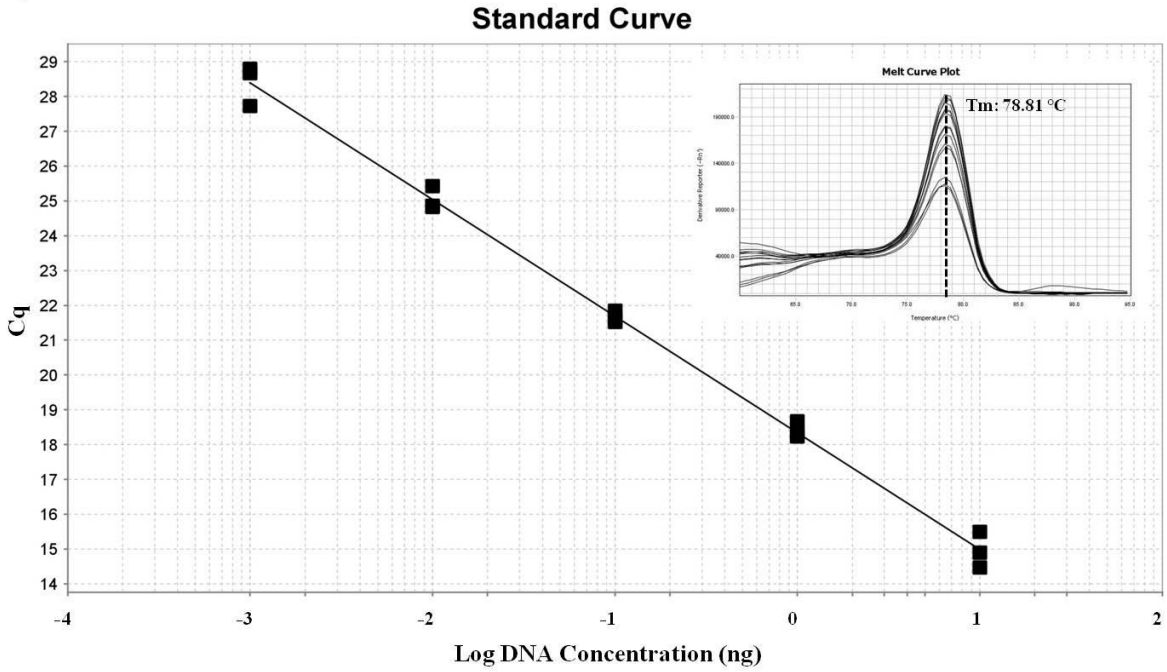
The standard curve for the skate-universal qPCR assay was plotted using either DNA extracted from *Beringraja pulchra* or a DNA mixture extracted from *O. kenojei*, *Dipturus chilensis*, and *Dipturus laevis*, as described. The efficiency, slope, and R^2 obtained for *Beringraja pulchra* samples were 95.6%, -3.432, and 0.999, respectively, while for the DNA mixture, these values were 98.8%, -3.351, and 0.999, respectively. The LOD obtained using the skate-universal qPCR was 0.001 ng/μL for both *Beringraja pulchra* DNA and the DNA mixture under the 30-cycle conditions (Figures 3.3A and 3.3B). The Tm values for *Beringraja pulchra* and other species samples were 76.59℃ and 78.81℃, respectively, and the sequences of the *Beringraja pulchra* PCR product (179 bp) showed 100% sequence identity to that of the COI gene of *Beringraja pulchra* (KR676448.1).

The specificity and cross-reactivity of the skate-universal qPCR assay were examined using the ray species, *U. aurantiacus*, and *Dasyatis akajei*. The average Cq values obtained for *Beringraja pulchra* and *O. kenojei* samples were 13.56 ± 0.12 and 13.77 ± 0.09, respectively. However, no amplification or false positive results were observed for the ray species samples, *U. aurantiacus* (34.77 ± 0.78) and *Dasyatis akajei* (38.77 ± 0.52), under the stringent 30-cycle conditions (Figure 3.3C). These results suggest that the developed qPCR assays can specifically identify *Beringraja pulchra* and other skate species.

A


Standard Curve
Melt Curve Plot
Tm: 76.59 °C
Cq
Log DNA Concentration (ng)

B


Standard Curve
Melt Curve Plot
Tm: 78.81 °C
Cq
Log DNA Concentration (ng)

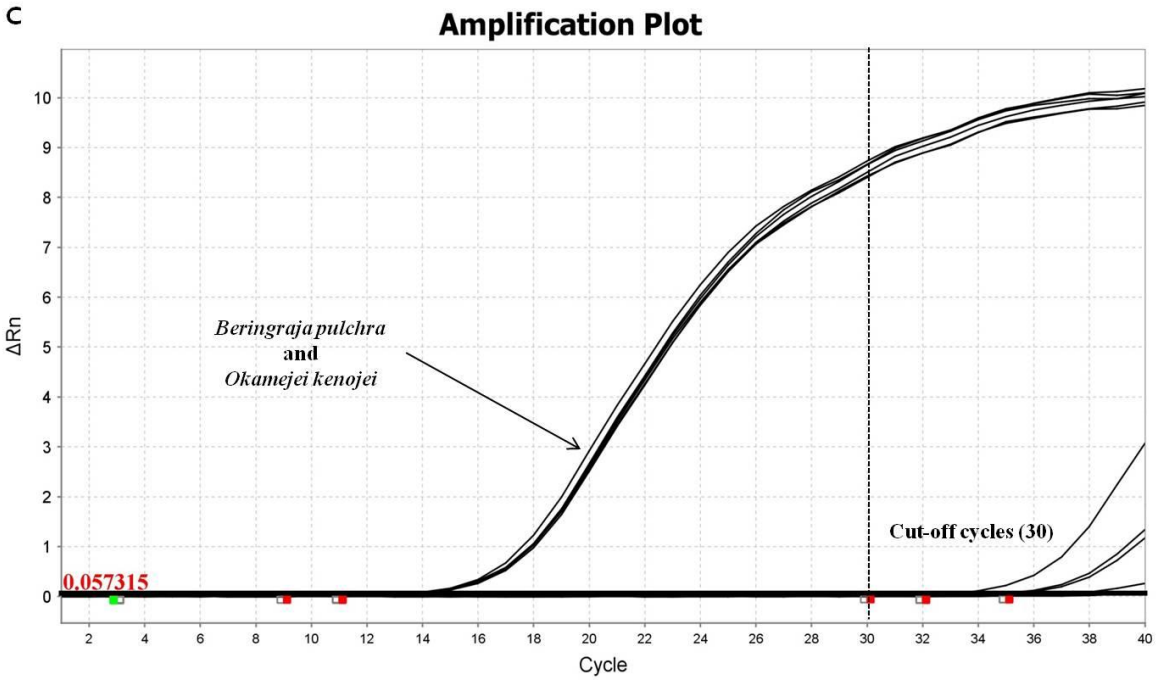


Figure 3.3 Standard curves and melting curves for the skate-universal qPCR assay using (A) *Beringraja pulchra* DNA and (B) a mixture of *O. kenojei*, *Dipturus chilensis*, and *Dipturus laevis* DNA. (C) Amplification pattern for *Beringraja pulchra* (Cq: 13.56 ± 0.12), *O. kenojei* (Cq: 13.77 ± 0.09), and ray species (Cq: 36.77 ± 2.77).

3.4.4 Identification of commercial skate products by FINS and qPCR assays

A total of twenty-six commercial skate products, all processed to different degrees (fresh, fermented, and seasoned), were analyzed in order to identify the skate species present and validate our qPCR assays. As discussed above, the COI sequence has been successfully used as a molecular marker for the genetic identification of skate species (Hwang, et al., 2015; Lago, Vieites, & Espineira, 2012; Spies, Gaichas, Stevenson, Orr, & Canino, 2006), and therefore, we also employed this to identify the skate species present in commercial products. Amplification of DNA extracted from all samples using the primers FISHF1 and FISHR2 generated a PCR product of approximately 655 bp (Figure 3.4), and subsequently, the PCR products were sequenced using the same primers. A BLAST search revealed 99 to 100% sequence identity for the PCR products to *Beringraja pulchra* (n = 16), *Dipturus chilensis* (n = 9), and *Dipturus laevis* (n = 1) in the GenBank database (Table 3.1). The commercial product labels declared only the general commercial name (*i.e.*, skate), but not the scientific names of the species. The FINS analysis demonstrated that ten samples declared to contain skate, which were imported from Argentina (n = 6), Chile (n = 1), or were of unknown origin (n = 3), principally contained two species, *Dipturus chilensis* and *Dipturus laevis*, whereas in sixteen samples labeled as using 100% domestic skate, *Beringraja pulchra* DNA was detected. This result was in accordance with the data obtained using our qPCR assays. The skate-universal qPCR results showed that the average Cq values obtained for all commercial samples ranged from 12 to 28 cycles, which were all significantly different from the values obtained for the ray species samples (34 to 38 cycles), suggesting that skate species were used in all products. The *Beringraja pulchra*-specific qPCR assay showed that the average Cq values obtained for *Beringraja pulchra* samples was 16.27 ± 1.40, which differed significantly from the average Cq values obtained for other skate and ray species (37.79 ±

2.16) ($P < 0.001$). Although slight signals, in the range of 34-38 cycles, were observed for the *O. kenojei* and *D. chilensis* samples, the much lower Cq values obtained for *Beringraja pulchra* were sufficient to discriminate this species from other skate species. Additionally, the Cq values obtained for the *Beringraja pulchra* samples here are comparable to those determined in previous qPCR-based fish species identification studies, including *Beringraja pulchra* (Hwang, et al., 2015), *Merluccius merluccius* (Sanchez, Quinteiro, Rey-Mendez, Perez-Martin, & Sotelo, 2009), *Gadus morhua* (Herrero, Madrinan, Vieites, & Espineira, 2010), *Molva* (Taboada, Sanchez, & Sotelo, 2017), and *Scomber scombrus* (Velasco, Sanchez, Martinez, Santaclara, Perez-Martin, & Sotelo, 2013), where positive and negative average Cq values ranged from 14 to 20 cycles and over 30 cycles, respectively. Therefore, these results demonstrate that the two newly developed qPCR assays, using the two primer sets, can be used to differentiate skates from rays, and *Beringraja pulchra* from the very closely-related skate species.

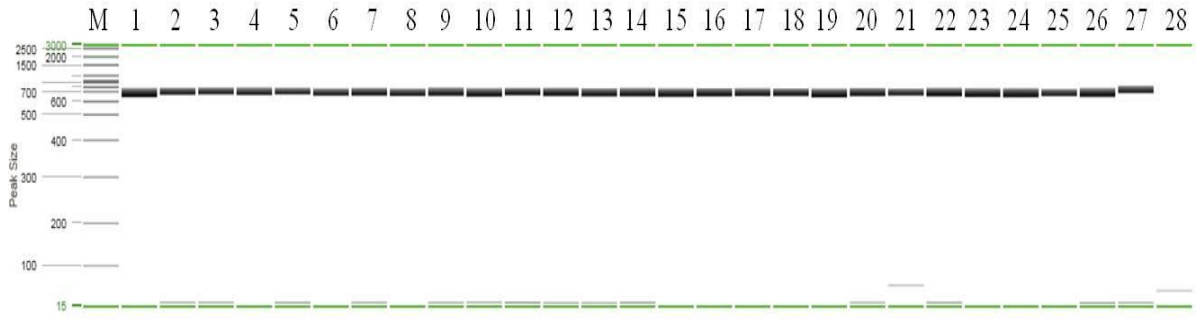


Figure 3.4 QIAxcel gel image of PCR products amplified from twenty-six commercial skate products. M: QX DNA size marker (100 bp to 2.5 kb), Lane 1: positive control (*Beringraja pulchra*), Lanes 2~27: the twenty-six commercial samples, and Lane 28: negative control.

3.4.5 Highly accurate qPCR assay based on the two primer sets

The Cq value is the most critical parameter that demonstrates the specificity and sensitivity of qPCR-based methods. Theoretically, Cq values are inversely proportional to the initial amount of target DNA. However, inhibitory substances and excipients in processed and functional foods can absorb DNA, hampering its extraction, thereby limiting its amplification (J. Costa, Amaral, Fernandes, Batista, Oliveira, & Mafra, 2015). Additionally, previous studies that analyzed highly-processed food have reported that qPCR results (*e.g.*, Cq value) depend significantly on the degree of processing, the seasoning and spices used, and the type of food preservatives added (Hwang, et al., 2015; Taboada, Sanchez, & Sotelo, 2017). As shown in Table 3.1, data from the skate-universal qPCR assay showed a significant difference in the Cq range between domestic (12 to 18 cycles) and imported skate species (15 to 28 cycles). This may be due to differences in distribution channels, processing, and/or fermentation. Therefore, to overcome this main drawback of qPCR, we developed a new, highly accurate method that utilizes differences in amplification efficiency between *Beringraja pulchra*-specific and skate-universal qPCR assays. As shown in Figure 3.5, when the two qPCR standard curves, obtained using *Beringraja pulchra* DNA samples, were plotted we noted that there was a tendency of constant deviation between the Cq values obtained from each linear regression equation wherein ΔCq represents a subtraction of the average Cq value using the skate-universal qPCR from the average Cq value using the *Beringraja pulchra*-specific qPCR. A linear regression equation ($y = 0.123x + 1.358$, $R^2 = 0.911$), calculated from the ΔCq values (1.54 to 1.01) and the different quantities of DNA (10 to 0.001 ng/μL), estimated that ΔCq values do not exceed 1.8 even if a 100-fold higher DNA concentration (1000 ng/μL) was used. When the skate DNA mixture (*O. kenojei*, *D. chilensis*, and *D. laevis*) was used instead, and the standard curve for the skate-universal qPCR assay

was plotted from the Cq values and the different DNA concentrations, a linear regression equation was obtained ($y = -3.351x + 18.340$, $R^2 = 0.999$).

It should be noted that, as expected, it was not possible to generate a standard curve using the *Beringraja pulchra*-specific qPCR assay with the skate DNA mixture (*O. kenojei*, *D. chilensis*, and *D. laevis*) as template due to the low efficiency of amplification. Based on this, we used our ΔCq method to analyze twenty-six commercial skate products. As shown in Table 3.1, we found a significant difference in the average ΔCq values obtained for *Beringraja pulchra* samples (1.18 ± 0.15) compared to samples of the other skate species (18.94 ± 3.46) ($P < 0.001$). Therefore, our method was shown to overcome the main drawback of previous qPCR protocols in the differentiation of *Beringraja pulchra* and other skate species in commercially-processed products.

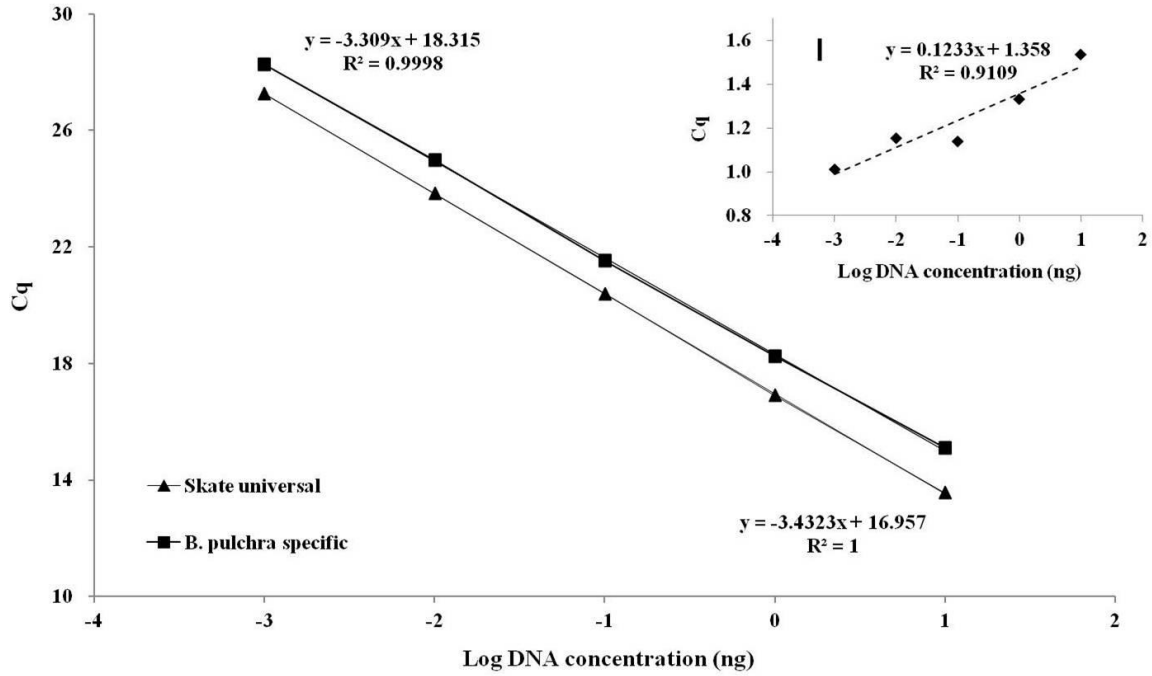


Figure 3.5 Comparison of *Beringraja pulchra*-specific and skate-universal standard curves obtained using *Beringraja pulchra* DNA. The insert "I" corresponds to the linear regression equation calculated from ΔCq values (1.54 to 1.01) and DNA quantities (10 to 0.001 ng/µL).

3.4.6 Ultra-fast qPCR assay for on-site applications

Our method was further improved to develop an ultra-fast qPCR assay for on-site application. Three aspects should be considered during the development of on-site methods based on PCR, including short DNA preparation, PCR operation, and data analysis time. Previously, loop-mediated isothermal amplification (LAMP) and convective PCR methods have been reported for the rapid molecular identification of genetically modified foods, plant materials, and meat products (Huang, Chen, Xu, Ji, Zhu, & Chen, 2014; Li, Wong, Jiang, Wong, Wong, Lau, et al., 2013; Song, Hwang, & Kim, 2017). PCR amplification step in these two methods can be completed within 20 to 30 min. However, DNA preparation for the LAMP assay and post-PCR analysis (*e.g.*, gel electrophoresis) in the convective PCR assay require extra time (20 to 60 min). For the development of an ultra-fast qPCR method, we used the Rapi:Prep system to extract DNA within 10 min and the GENECHECKER Ultra-Fast Real-Time PCR system with a special polymer chip (Rapi:chip) allowing for fast thermal changes (8℃/s ramping rate for both heating and cooling) and an integrated camera module for fluorescence detection, enabling qPCR reaction and data analysis in 15 min. The twenty-six commercial skate products were analyzed using this ultra-fast qPCR method under optimal conditions (Table 3.3). The utility of this ultra-fast qPCR method was assessed by examining amplification curves, melting curves, and ΔCq values (Figure 3.6), which were shown to agree well with the data obtained using the qPCR assays (Table 3.1).

Therefore, we successfully demonstrated that the entire analytical procedure, including DNA isolation, qPCR amplification, and detection, can be completed within 30 min using our ultra-fast qPCR method, maintaining the accuracy of *Beringraja pulchra*, skate, and ray identification (*i.e.*, an average ΔCq value < 3.0 indicates the presence of *Beringraja pulchra*, an average ΔCq value > 10.0 indicates the presence of other skate species, while an

average Cq value > 30 indicates the presence of ray species). Additionally, considering that GENECHECKER is a battery-operated and hand-held portable device, our method represents an appropriate tool for the authentication of skate products on the spot.

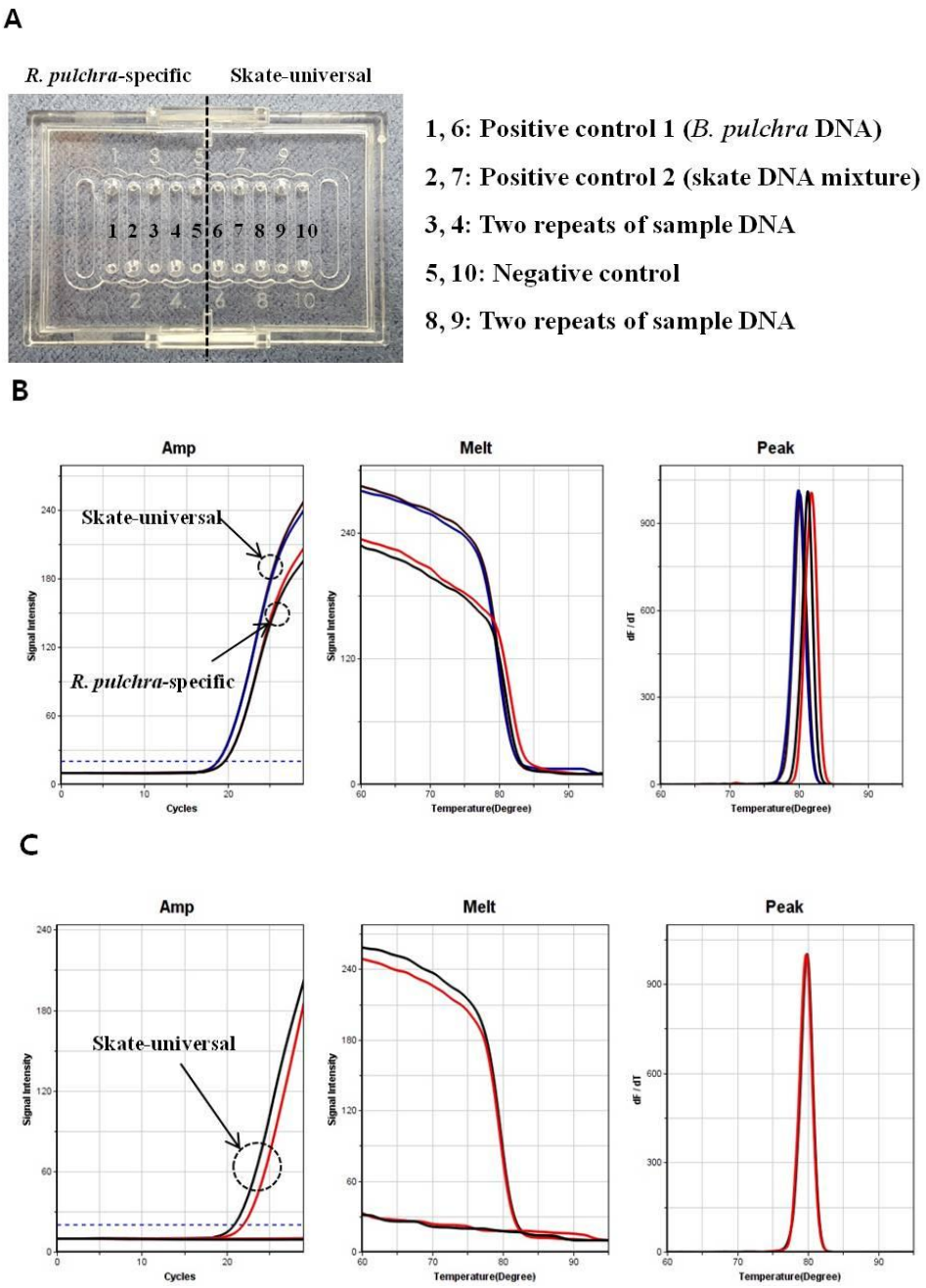


Figure 3.6 (A) Schematic representation of the ultra-fast qPCR assay. Sample layout on the polymer chip (Rapi:chip) for the ultra-fast qPCR of skate products, real-time amplification plots, and melting curves of (B) *Beringraja pulchra* and (C) *Dipturus chilensis* samples. The average Cq value of each assay was obtained from two replicates.

3.5 Conclusions

In this study, we designed *Beringraja pulchra*-specific and skate-universal primer sets and developed an accurate qPCR assay based on these two primer sets for the identification of skate product components. Our method overcomes some of the current limitations of qPCR techniques for the testing of highly-processed food and can successfully assess the authenticity of commercial skate products. In addition, we developed an ultra-fast qPCR method for efficient on-site analyses that requires a significantly shorter analysis time (less than 30 min) than other rapid methods. Therefore, our method can be efficiently utilized to verify the authenticity of raw and processed products in the field, providing an accurate estimate of the actual content of *Beringraja pulchra* products. Additionally, in order to protect the consumer's rights, this method can be easily extended to develop advanced detection methods for the regulation of fraudulent practices in the food industry.

4. References

Alonso-Rebollo, A., Ramos-Gomez, S., Busto, M. D., & Ortega, N. (2017). Development and optimization of an efficient qPCR system for olive authentication in edible oils. *Food Chemistry, 232*, 827-835.

Amaral, J. S., Santos, C. G., Melo, V. S., Oliveira, M. B. P. P., & Mafra, I. (2014). Authentication of a traditional game meat sausage (Alheira) by species-specific PCR assays to detect hare, rabbit, red deer, pork and cow meats. *Food Research International, 60*, 140-145.

Amaral, J. S., Santos, G., Oliveira, M. B. P. P., & Mafra, I. (2017). Quantitative detection of pork meat by EvaGreen real-time PCR to assess the authenticity of processed meat products. *Food Control, 72*, 53-61.

Arya, M., Shergill, I. S., Williamson, M., Gommersall, L., Arya, N., & Patel, H. R. (2005). Basic principles of real-time quantitative PCR. *Expert Review of Molecular Diagnostics, 5*(2), 209-219.

Ballin, N. Z., Vogensen, F. K., & Karlsson, A. H. (2009). Species determination - Can we detect and quantify meat adulteration? *Meat Science, 83*(2), 165-174.

Bartlett, S. E., & Davidson, W. S. (1991). Identification of *Thunnus* tuna species by the polymerase chain reaction and direct sequence analysis of their mitochondrial cytochrome b genes. *Canadian Journal of Fisheries and Aquatic Sciences, 48*(2), 309-317.

Bartlett, S. E., & Davidson, W. S. (1992). FINS (forensically informative nucleotide sequencing): a procedure for identifying the animal origin of biological specimens. *Biotechniques, 12*(3), 408-411.

Broeders, S., Huber, I., Grohmann, L., Berben, G., Taverniers, I., Mazzara, M., Roosens, N.,

& Morisset, D. (2014). Guidelines for validation of qualitative real-time PCR methods. *Trends in Food Science & Technology, 37*(2), 115-126.

Burns, M., & Valdivia, H. (2008). Modelling the limit of detection in real-time quantitative PCR. *European Food Research and Technology, 226*(6), 1513-1524.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry, 55*(4), 611-622.

Cawthorn, D.-M., Steinman, H. A., & Witthuhn, R. C. (2011). Comparative study of different methods for the extraction of DNA from fish species commercially available in South Africa. *Food Control, 22*(2), 231-244.

Cho, Y. A., Kim, E.-M., Kim, M.-J., Kang, J.-H., Dong, C. M., An, H. S., An, C.-M., Park, M. A., & Park, J. Y. (2014). A rapid and simple method for distinguishing two mitten crabs (*Eriocheir sinensis* and *Eriocheir japonica*) in Korea using PCR-RFLP and PCR. *Food Control, 36*(1), 20-23.

Chuah, L.-O., He, X. B., Effarizah, M. E., Syahariza, Z. A., Shamila-Syuhada, A. K., & Rusul, G. (2016). Mislabelling of beef and poultry products sold in Malaysia. *Food Control, 62*, 157-164.

Costa, J., Amaral, J. S., Fernandes, T. J., Batista, A., Oliveira, M. B., & Mafra, I. (2015). DNA extraction from plant food supplements: Influence of different pharmaceutical excipients. *Mol Cell Probes, 29*(6), 473-478.

Costa, J., Amaral, J. S., Grazina, L., Oliveira, M., & Mafra, I. (2017). Matrix-normalised real-time PCR approach to quantify soybean as a potential food allergen as affected by thermal processing. *Food Chemistry, 221*, 1843-1850.

Costa, J., Oliveira, M. B. P. P., & Mafra, I. (2013). Effect of thermal processing on the

performance of the novel single-tube nested real-time PCR for the detection of walnut allergens in sponge cakes. *Food Research International, 54*(2), 1722-1729.

D'Andrea, M., Coïsson, J. D., Locatelli, M., Garino, C., Cereti, E., & Arlorio, M. (2011). Validating allergen coding genes (Cor a 1, Cor a 8, Cor a 14) as target sequences for hazelnut detection via Real-Time PCR. *Food Chemistry, 124*(3), 1164-1171.

de la Cruz, S., López-Calleja, I. M., Alcocer, M., González, I., Martín, R., & García, T. (2013). TaqMan real-time PCR assay for detection of traces of Brazil nut (*Bertholletia excelsa*) in food products. *Food Control, 33*(1), 105-113.

Demeke, T., Malabanan, J., Holigroski, M., & Eng, M. (2017). Effect of Source of DNA on the Quantitative Analysis of Genetically Engineered Traits Using Digital PCR and Real-Time PCR. *Journal of AOAC International, 100*(2), 492-498.

Di Pinto, A., Terio, V., Marchetti, P., Bottaro, M., Mottola, A., Bozzo, G., Bonerba, E., Ceci, E., & Tantillo, G. (2000). DNA-based approach for species identification of goat-milk products. *Food Chemistry, 229*, 93-97.

Druml, B., Grandits, S., Mayer, W., Hochegger, R., & Cichna-Markl, M. (2015). Authenticity control of game meat products-a single method to detect and quantify adulteration of fallow deer (*Dama dama*), red deer (*Cervus elaphus*) and sika deer (*Cervus nippon*) by real-time PCR. *Food Chemistry, 170*, 508-517.

Druml, B., Hochegger, R., & Cichna-Markl, M. (2015). Duplex real-time PCR assay for the simultaneous determination of the roe deer (*Capreolus capreolus*) and deer (sum of fallow deer, red deer and sika deer) content in game meat products. *Food Control, 57*, 370-376.

Druml, B., Kaltenbrunner, M., Hochegger, R., & Cichna-Markl, M. (2016). A novel reference real-time PCR assay for the relative quantification of (game) meat species in raw and heat-processed food. *Food Control, 70*, 392-400.

Druml, B., Mayer, W., Cichna-Markl, M., & Hochegger, R. (2015). Development and validation of a TaqMan real-time PCR assay for the identification and quantification of roe deer (*Capreolus capreolus*) in food to detect food adulteration. *Food Chemistry, 178*, 319-326.

ENGL (European Network of GMO Laboratories). Definition of minimum performance requirements for analytical methods of GMO testing. (2015). http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020_10_2015.pdf/ Accessed 21 December 2017.

Espineira, M., & Vieites, J. M. (2012). Rapid method for controlling the correct labeling of products containing common octopus (*Octopus vulgaris*) and main substitute species (*Eledone cirrhosa* and *Dosidicus gigas*) by fast real-time PCR. *Food Chemistry, 135*(4), 2439-2444.

Eugster, A., Ruf, J., Rentsch, J., Hübner, P., & Köppel, R. (2008). Quantification of beef and pork fraction in sausages by real-time PCR analysis: results of an interlaboratory trial. *European Food Research and Technology, 227*(1), 17-20.

Eugster, A., Ruf, J., Rentsch, J., & Köppel, R. (2009). Quantification of beef, pork, chicken and turkey proportions in sausages: use of matrix-adapted standards and comparison of single versus multiplex PCR in an interlaboratory trial. *European Food Research and Technology, 230*(1), 55-61.

EURL-AP (European Union Reference Laboratory for Animal Proteins). Addendum to the EURL-AP protocol : Cut-off to check the 1% level threshold of horse meat in another meat. (2013). http://www.innofoodsee.eu/downloads/addendum_protocol_horse_detection.pdf/ Accessed 21 December 2017.

Fajardo, V., Gonzalez, I., Martin, I., Rojas, M., Hernandez, P. E., Garcia, T., & Martin, R.

(2008). Real-time PCR for detection and quantification of red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*) in meat mixtures. *Meat Science, 79*(2), 289-298.

Fang, X., & Zhang, C. (2016). Detection of adulterated murine components in meat products by TaqMan(c) real-time PCR. *Food Chemistry, 192*, 485-490.

Fernandes, T. J. R., Costa, J., Oliveira, M. B. P. P., & Mafra, I. (2017). Exploiting 16S rRNA gene for the detection and quantification of fish as a potential allergenic food: A comparison of two real-time PCR approaches. *Food Chemistry, https://doi.org/10.1016/j.foodchem.2017.11.068*.

Ferreira, T., Farah, A., Oliveira, T. C., Lima, I. S., Vitorio, F., & Oliveira, E. M. (2016). Using Real-Time PCR as a tool for monitoring the authenticity of commercial coffees. *Food Chemistry, 199*, 433-438.

Fuchs, M., Cichna-Markl, M., & Hochegger, R. (2012). Development and validation of a novel real-time PCR method for the detection of celery (*Apium graveolens*) in food. *Food Chemistry, 130*(1), 189-195.

Fuchs, M., Cichna-Markl, M., & Hochegger, R. (2013). Development and validation of a duplex real-time PCR method for the simultaneous detection of celery and white mustard in food. *Food Chemistry, 141*(1), 229-235.

Giaretta, N., Di Giuseppe, A. M., Lippert, M., Parente, A., & Di Maro, A. (2013). Myoglobin as marker in meat adulteration: a UPLC method for determining the presence of pork meat in raw beef burger. *Food Chemistry, 141*(3), 1814-1820.

Hellberg, R. S., & Morrissey, M. T. (2011). Advances in DNA-based techniques for the detection of seafood species substitution on the commercial market. *Journal of Laboratory Automation, 16*(4), 308-321.

Herrero, B., Madrinan, M., Vieites, J. M., & Espineira, M. (2010). Authentication of Atlantic

cod (*Gadus morhua*) using real time PCR. *Journal of Agricultural and Food Chemistry, 58*(8), 4794-4799.

Herrero, B., Vieites, J. M., & Espineira, M. (2014). Development of an in-house fast real-time PCR method for detection of fish allergen in foods and comparison with a commercial kit. *Food Chemistry, 151*, 415-420.

Holzhauser, T., Kleiner, K., Janise, A., & Roder, M. (2014). Matrix-normalised quantification of species by threshold-calibrated competitive real-time PCR: allergenic peanut in food as one example. *Food Chemistry, 163*, 68-76.

Hossain, M. A. M., Ali, M. E., Sultana, S., Asing, Bonny, S. Q., Kader, M. A., & Rahman, M. A. (2017). Quantitative Tetraplex Real-Time Polymerase Chain Reaction Assay with TaqMan Probes Discriminates Cattle, Buffalo, and Porcine Materials in Food Chain. *Journal of Agricultural and Food Chemistry, 65*(19), 3975-3985.

Hu, Y., Zou, L., Huang, X., & Lu, X. (2017). Detection and quantification of offal content in ground beef meat using vibrational spectroscopic-based chemometric analysis. *Scientific reports, 7*(1), DOI: 10.1038/s41598-41017-15389-41593.

Huang, X., Chen, L., Xu, J., Ji, H. F., Zhu, S., & Chen, H. (2014). Rapid visual detection of phytase gene in genetically modified maize using loop-mediated isothermal amplification method. *Food Chemistry, 156*, 184-189.

Hwang, I. K., Lee, H. Y., Kim, M. H., Jo, H. S., Choi, D. H., Kang, P. W., Lee, Y. H., Cho, N. S., Park, K. W., & Chae, H. Z. (2015). Development of real-time PCR assay for genetic identification of the mottled skate, *Beringraja pulchra*. *Forensic Science International, 255*, 80-84.

ICH (The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use). Validation of analytical procedures: text and methodoloty Q2 (R1). (2005).

https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf/ Accessed 21 December 2017.

Iniesto, E., Jimenez, A., Prieto, N., Cabanillas, B., Burbano, C., Pedrosa, M. M., Rodriguez, J., Muzquiz, M., Crespo, J. F., Cuadrado, C., & Linacero, R. (2013). Real Time PCR to detect hazelnut allergen coding sequences in processed foods. *Food Chemistry, 138*(2-3), 1976-1981.

IUCN (International Union for Conservation of Nature). The IUCN Red List of Threatened Species, 2017. URL: http://dx.doi.org/10.2305/IUCN.UK.2009-.RLTS.T161669A5476757.en. Accessed 24.08.17.

Iwobi, A., Sebah, D., Kraemer, I., Losher, C., Fischer, G., Busch, U., & Huber, I. (2015). A multiplex real-time PCR method for the quantification of beef and pork fractions in minced meat. *Food Chemistry, 169*, 305-313.

Iwobi, A., Sebah, D., Spielmann, G., Maggipinto, M., Schrempp, M., Kraemer, I., Gerdes, L., Busch, U., & Huber, I. (2017). A multiplex real-time PCR method for the quantitative determination of equine (horse) fractions in meat products. *Food Control, 74*, 89-97.

Jeong, D., Kim, S., Kim, C. G., Myoung, J. G., & Lee, Y. H. (2014). Complete mitochondrial genome of the mottled skate: Raja pulchra *(Rajiformes, Rajidae*). *Mitochondrial DNA, Part A: DNA Mapping, Sequencing, and Analysis*, *27*(3), 1823-1824.

Kaltenbrunner, M., Hochegger, R., & Cichna-Markl, M. (2018). Development and validation of a fallow deer (*Dama dama*)-specific TaqMan real-time PCR assay for the detection of food adulteration. *Food Chemistry, 243*, 82-90.

Kane, D. E., & Hellberg, R. S. (2016). Identification of species in ground meat products sold on the US commercial market using DNA-based methods. *Food Control, 59*, 158-163.

Kang, J. H., Park, J. Y., & Jo, H. S. (2012). Rapid development of microsatellite markers with 454 pyrosequencing in a vulnerable fish, the mottled skate, *Raja pulchra*.

International Journal of Molecular Sciences, 13(6), 7199-7211.

Kempter, J., Kielpinski, M., Panicz, R., Pruffer, K., & Keszka, S. (2017). Development of the method for identification of selected populations of torpedo scad, *Megalaspis cordyla* (Linnaeus, 1758), using microsatellite DNA analyses. CELFISH project - Part 4. *Food Chemistry, 221*, 944-949.

Kim, J. H., Moon, J.-C., Kang, T. S., Kwon, K., & Jang, C. S. (2017). Development of cpDNA markers for discrimination between *Cynanchum wilfordii* and *Cynanchum auriculatum* and their application in commercial *C. wilfordii* food products. *Applied Biological Chemistry, 60*(1), 79-86.

Kim, M.-j., & Kim, H.-Y. (2018). Development of a fast duplex real-time PCR assay for simultaneous detection of chicken and pigeon in raw and heat-treated meats. *Food Control, 85*, 1-5.

Kim, M.-R., Kwon, K., Jung, Y.-K., & Kang, T. S. (2018). A rapid real-time PCR method to differentiate between mottled skate (*Beringraja pulchra*) and other skate and ray species. *Food Chemistry*, https://doi.org/10.1016/j.foodchem.2018.1002.1056.

Kim, M., Yoo, I., Lee, S.-Y., Hong, Y., & Kim, H.-Y. (2016). Quantitative detection of pork in commercial meat products by TaqMan real-time PCR assay targeting the mitochondrial D-loop region. *Food Chemistry, 210*, 102-106.

Kitano, T., Umetsu, K., Tian, W., & Osawa, M. (2007). Two universal primer sets for species identification among vertebrates. *International Journal of Legal Medicine, 121*(5), 423-427.

Kralik, P., & Ricchi, M. (2017). A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. *Frontiers in Microbiology, 8*, 108.

López-Andreo, M., Aldeguer, M., Guillén, I., Gabaldón, J. A., & Puyet, A. (2012). Detection and quantification of meat species by qPCR in heat-processed food containing highly

fragmented DNA. *Food Chemistry, 134*(1), 518-523.

López-Andreo, M., Lugo, L., Garrido-Pertierra, A., Prieto, M. I., & Puyet, A. (2005). Identification and quantitation of species in complex DNA mixtures by real-time polymerase chain reaction. *Analytical Biochemistry, 339*(1), 73-82.

López-Calleja, I. M., de la Cruz, S., Gonzalez, I., Garcia, T., & Martin, R. (2015). Market analysis of food products for detection of allergenic walnut (*Juglans regia*) and pecan (*Carya illinoinensis*) by real-time PCR. *Food Chemistry, 177*, 111-119.

López-Calleja, I. M., de la Cruz, S., Pegels, N., González, I., García, T., & Martín, R. (2013). High resolution TaqMan real-time PCR approach to detect hazelnut DNA encoding for ITS rDNA in foods. *Food Chemistry, 141*(3), 1872-1880.

Lago, F. C., Vieites, J. M., & Espineira, M. (2012). Development of a FINS-based method for the identification of skates species of commercial interest. *Food Control, 24*(1), 38-43.

Laube, I., Zagon, J., & Broll, H. (2007). Quantitative determination of commercially relevant species in foods by real-Iime PCR. *International Journal of Food Science & Technology, 42*(3), 336-341.

Laube, I., Zagon, J., Spiegelberg, A., Butschke, A., Kroh, L. W., & Broll, H. (2007). Development and design of a 'ready-to-use' reaction plate for a PCR-based simultaneous detection of animal species used in foods. *International Journal of Food Science & Technology, 42*(1), 9-17.

Lee, J. H., Kim, M. R., Jo, C. H., Jung, Y. K., Kwon, K., & Kang, T. S. (2016). Specific PCR assays to determine bovine, porcine, fish and plant origin of gelatin capsules of dietary supplements. *Food Chemistry, 211*, 253-259.

Li, M., Wong, Y. L., Jiang, L. L., Wong, K. L., Wong, Y. T., Lau, C. B., & Shaw, P. C. (2013). Application of novel loop-mediated isothermal amplification (LAMP) for rapid authentication of the herbal tea ingredient *Hedyotis diffusa* Willd. *Food Chemistry,*

141(3), 2522-2525.

Macedo-Silva, A., Barbosa, S. F., Alkmin, M. G., Vaz, A. J., Shimokomaki, M., & Tenuta-Filho, A. (2000). Hamburger meat identification by dot-ELISA. *Meat Science, 56*(2), 189-192.

Maede, D. (2006). A strategy for molecular species detection in meat and meat products by PCR-RFLP and DNA sequencing using mitochondrial and chromosomal genetic sequences. *European Food Research and Technology, 224*(2), 209-217.

Mano, J., Nishitsuji, Y., Kikuchi, Y., Fukudome, S. I., Hayashida, T., Kawakami, H., Kurimoto, Y., Noguchi, A., Kondo, K., Teshima, R., Takabatake, R., & Kitta, K. (2017). Quantification of DNA fragmentation in processed foods using real-time PCR. *Food Chemistry, 226*, 149-155.

Meira, L., Costa, J., Villa, C., Ramos, F., Oliveira, M. B. P. P., & Mafra, I. (2017). EvaGreen real-time PCR to determine horse meat adulteration in processed foods. *LWT-Food Science and Technology, 75*, 408-416.

Moore, J. C., Spink, J., & Lipp, M. (2012). Development and application of a database of food ingredient fraud and economically motivated adulteration from 1980 to 2010. *J Food Sci, 77*(4), 118-126.

Moyer, D. C., DeVries, J. W., & Spink, J. (2017). The economics of a food fraud incident - Case studies and examples including Melamine in Wheat Gluten. *Food Control, 71*, 358-364.

Mujico, J. R., Lombardía, M., Mena, M. C., Méndez, E., & Albar, J. P. (2011). A highly sensitive real-time PCR system for quantification of wheat contamination in gluten-free food for celiac patients. *Food Chemistry, 128*(3), 795-801.

Naaum, A. M., Shehata, H. R., Chen, S., Li, J., Tabujara, N., Awmack, D., Lutze-Wallace, C., & Hanner, R. (2018). Complementary molecular methods detect undeclared species in

sausage products at retail markets in Canada. *Food Control*, *84*, 339-344.

Pafundo, S., Gullì, M., & Marmiroli, N. (2009). SYBR® GreenER™ Real-Time PCR to detect almond in traces in processed food. *Food Chemistry, 116*(3), 811-815.

Palle-Reisch, M., Hochegger, R., & Cichna-Markl, M. (2015). Development and validation of a triplex real-time PCR assay for the simultaneous detection of three mustard species and three celery varieties in food. *Food Chemistry, 184*, 46-56.

Pegels, N., González, I., Martín, I., Rojas, M., García, T., & Martín, R. (2011). Applicability assessment of a real-time PCR assay for the specific detection of bovine, ovine and caprine material in feedstuffs. *Food Control*, *22*(8), 1189-1196.

Pegels, N., Gonzalez, I., Garcia, T., & Martin, R. (2014). Avian-specific real-time PCR assay for authenticity control in farm animal feeds and pet foods. *Food Chemistry, 142*, 39-47.

Ramos-Gomez, S., Busto, M. D., Albillos, S. M., & Ortega, N. (2016). Novel qPCR systems for olive (*Olea europaea* L.) authentication in oils and food. *Food Chemistry, 194*, 447-454.

Rasmussen, R. S., & Morrissey, M. T. (2008). DNA-based methods for the identification of commercial fish and seafood species. *Comprehensive Reviews in Food science and Food Safety, 7*(3), 280-295.

Rojas, M., Gonzalez, I., Pavon, M. A., Pegels, N., Lago, A., Hernandez, P. E., Garcia, T., & Martin, R. (2010). Novel TaqMan real-time polymerase chain reaction assay for verifying the authenticity of meat and commercial meat products from game birds. *Food Additives & Contaminants Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 27*(6), 749-763.

Sambrook, J., & Russell, D. (2001). *Molecular cloning: a laboratory manual* (3rd ed.). New York: Cold Spring Harbor Laboratory Press.

Sanchez, A., Quinteiro, J., Rey-Mendez, M., Perez-Martin, R. I., & Sotelo, C. G. (2009). Identification of European Hake species (*Merluccius merluccius*) using real-time PCR. *Journal of Agricultural and Food Chemistry, 57*(9), 3397-3403.

Scarafoni, A., Ronchi, A., & Duranti, M. (2009). A real-time PCR method for the detection and quantification of lupin flour in wheat flour-based matrices. *Food Chemistry, 115*(3), 1088-1093.

Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors - occurrence, properties and removal. *Journal of Applied Microbiology, 113*(5), 1014-1026.

Soares, S., Amaral, J. S., Oliveira, M. B., & Mafra, I. (2013). A SYBR Green real-time PCR assay to detect and quantify pork meat in processed poultry meat products. *Meat Science, 94*(1), 115-120.

Song, K. Y., Hwang, H. J., & Kim, J. H. (2017). Ultra-fast DNA-based multiplex convection PCR method for meat species identification with possible on-site applications. *Food Chemistry, 229*, 341-346.

Spies, I. B., Gaichas, S., Stevenson, D. E., Orr, J. W., & Canino, M. F. (2006). DNA-based identification of Alaska skates (*Amblyraja*, *Bathyraja* and *Raja*: *Rajidae*) using cytochrome c oxidase subunit I (coI) variation. *Journal of Fish Biology, 69*(sb), 283-292.

Taboada, L., Sanchez, A., & Sotelo, C. G. (2017). A new real-time PCR method for rapid and specific detection of ling (*Molva molva*). *Food Chemistry, 228*, 469-475.

Tagliavia, M., Nicosia, A., Salamone, M., Biondo, G., Bennici, C. D., Mazzola, S., & Cuttitta, A. (2016). Development of a fast DNA extraction method for sea food and marine species identification. *Food Chemistry, 203*, 375-378.

Velasco, A., Sanchez, A., Martinez, I., Santaclara, F. J., Perez-Martin, R. I., & Sotelo, C. G. (2013). Development of a real-time PCR method for the identification of Atlantic

mackerel (*Scomber scombrus*). *Food Chemistry, 141*(3), 2006-2010.

Villa, C., Costa, J., Oliveira, M. B., & Mafra, I. (2017). Novel quantitative real-time PCR approach to determine safflower (*Carthamus tinctorius*) adulteration in saffron (*Crocus sativus*). *Food Chemistry, 229*, 680-687.

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R., & Hebert, P. D. (2005). DNA barcoding Australia's fish species. *Philosophical transactions of the Royal Society of London, 360*(1462), 1847-1857.

Wen, J., Hu, C., Zhang, L., & Fan, S. (2012). Identification of 11 sea cucumber species by species-specific PCR method. *Food Control, 27*(2), 380-384.

Wilwet, L., Jeyasekaran, G., Shakila, R. J., Sivaraman, B., & Padmavathy, P. (2017). A single enzyme PCR-RFLP protocol targeting 16S rRNA/tRNA^{val} region to authenticate four commercially important shrimp species in India. *Food Chemistry, 239*, 369-376.

Yalcinkaya, B., Yumbul, E., Mozioglu, E., & Akgoz, M. (2017). Comparison of DNA extraction methods for meat analysis. *Food Chemistry, 221*, 1253-1257.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics, 13*(134), DOI: 10.1186/1471-2105-1113-1134.

Yuan, J. S., Reed, A., Chen, F., & Stewart, C. N., Jr. (2006). Statistical analysis of real-time PCR data. *BMC Bioinformatics, 7*(85), DOI: 10.1186/1471-2105-1187-1185.

Zagon, J., Schmidt, J., Schmidt, A. S., Broll, H., Lampen, A., Seidler, T., & Braeuning, A. (2017). A novel screening approach based on six real-time PCR systems for the detection of crustacean species in food. *Food Control, 79*, 27-34.

Zhang, W. J., Cai, Q., Guan, X., & Chen, Q. (2015). Detection of peanut (*Arachis hypogaea*) allergen by Real-time PCR method with internal amplification control. *Food Chemistry, 174*, 547-552.