Food Chemistry: Molecular Sciences 6 (2023) 100162

Contents lists available at ScienceDirect

Food Chemistry: Molecular Sciences

journal homepage: www.sciencedirect.com/journal/food-chemistry-molecular-sciences

Rapid detection of duck ingredient in adulterated foods by isothermal recombinase polymerase amplification assays

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ARTICLE INFO

Keywords:
Duck ingredient
Meat fraud
Cytb gene
Real-time RPA
LFS RPA
Rapid detection

ABSTRACT

Duck is often used in meat fraud as a substitute for more expensive meats. Rapid detection of duck ingredient in meat products is of great significance for combating meat fraud and safeguarding the interests of consumers. Therefore, we aim to develop duck-specific recombinase polymerase amplification (RPA)-based assays for the rapid detection of duck ingredient in animal-derived foods. Using *Cytb* gene as target, the real-time RPA and RPA combined with lateral flow strips (LFS RPA) were developed successfully for the rapid detection of ducks in 20 min at 39 °C and 40 °C, respectively. The assays did not show cross-reactions with 6 other livestock and poultry. The developed RPA assays could detect 10 pg duck genomic DNA per reaction and 0.1 % (w/w) duck ingredient in duck and mutton mixed powder within 30 min, including a rapid nucleic acid extraction. Furthermore, duck ingredient could be detected in 30 different actual foods including heat-processed meats and blood products. Therefore, duck-specific real-time RPA and LFS RPA assays were successfully developed with good specificity and sensitivity, which could enable rapid detection of duck ingredient in the field and provide technical support for combating the meat fraud.

1. Introduction

Economically Motivated Adulteration (EMA), a subcategory of food fraud, is defined as:“ … the fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production”(Spink & Moyer, 2011). EMA seriously affects the relationship between consumers and the food industry and undermines the public confidence in the entire food industry. EMA in meat foods corresponds to the substitution or the adulteration of more expensive meat with cheaper meat to make enormous profits, in other words, meat fraud. Previous market studies in China, U.S, Canada and Greek suggested that the meat fraud was a worldwide problem (Kane & Hellberg, 2016; Shehata et al., 2019; Song, Chen, Zhao, Ouyang, & Song, 2019; Stamatis et al., 2015). The meat fraud may result in serious public health consequences when the adulterant is toxic. For example, the horse meat disguised as beef in the European horsemeat scandal was detected to contain phenylbutazone, which is harmful to humans even in trace amounts (Abbas et al., 2018). Even undeclared adulterants could negatively affect health-related aspects (Bansal, Singh, Mangal, Mangal, & Kumar, 2017) and religious diets (Hossain et al., 2022). Therefore, adulteration identification plays an important role in the economic area and consumer’s physical and mental health.

Duck is one of the common species used as adulteration for lamb and beef because of their similar texture and color (Fu, Zhang, Zhou, & Liu, 2020; Qin et al., 2019; Zheng, Li, Wei, & Peng, 2019), especially the beef/lamb skewers and slices in barbecue stalls and hot pot restaurants in China. Duck-derived products are difficult for consumers to distinguish from beef and lamb due to using additives such as the butter to mask the smell and the beef or mutton essence to smell like them (Chen et al., 2022). Therefore, it is necessary to establish a simple, rapid, and sensitive method to detect duck ingredient in meat-related products.

Several methods have been developed for meat adulteration identification, including chromatography (Di Stefano et al., 2012) and spectroscopy (Weng et al., 2020). However, their application in the food industry for routine analysis is rarely discussed. Currently, the primary

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https://doi.org/10.1016/j.fochms.2023.100162
Received 8 November 2022; Received in revised form 3 January 2023; Accepted 7 January 2023
Available online 9 January 2023
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techniques for meat identification and adulteration are protein-based methods (Mandli, El Fatimi, Seddaoui, & Amine, 2018; Yamasaki et al., 2021) and DNA-based methods (Chen, Wei, Chen, Zhao, & Yang, 2015; Liu et al., 2019). Polymerase Chain Reaction (PCR)-based methods and enzyme-linked immunosorbent assay (ELISA) are the two main methods to detect meat adulteration (Konduru, Sagi, & Parida, 2021; Yin et al., 2020), and the former was extensively studied in the literature. ELISA is less expensive and time-saving, but it cannot discriminate between closely-related species (Li et al., 2020). The target protein denaturation caused by harsh processing conditions (Zhao et al., 2020) and the poor reproducibility for frozen products (Zia, Alawami, Mokhtar, Nhari, & Hanish, 2020) also hinder the ELISA application in processed meat products. PCR-based methods, especially real-time PCR, are more optimal for species identification in processed meat products because of the DNA thermostability. However, each procedure of the PCR method requires precise temperature control, so sophisticated equipment is indispensable, which makes it inconvenient to use outside the laboratory.

Nucleic acid isothermal amplification techniques, which require only a single constant temperature and are independent of thermal cycling instrument. Several promising isothermal amplification methods have emerged, while some of them need relatively high temperatures and/or a phase for complex primers design. For example, loop-mediated isothermal amplification (LAMP) requires 6–8 primers targeting different regions of the amplified fragment and working at 65 °C (Cai, Kong, & Xu, 2020) and cross-priming amplification (CPA) relies on 5 primers to detect the target sequence at 63 °C (Feng, Li, Wang, & Pan, 2018). In contrast, the RPA method works successfully over a wide temperature ranging from 37 ~ 42 °C with a specific primer pair (Yogesh Kumar, 2021). Thus, the amplification could be achieved even at room or human body temperature (Cherkaoui, Huang, Miller, Turbe, & McKendry, 2021).

RPA reaction primarily relies on three pivotal enzymes: recombinase, DNA polymerase and single-stranded DNA-binding protein (SSB). An oligonucleotide-protein complex consisting of primers and recombinase browses the template for homologous sequences. Then, the complex separates, a polymerase with strand-displacing activity binds the primers to the template, and the extension begins while SSB stabilizes another single strand. The amplification is initiated simultaneously from the forward and reverse primers to form a new double strand. Once this reaction is initiated, the template amplifies exponentially within 30 min. Amplification at constant temperature allows RPA to be used in conjunction with portable detection equipment, making detection simpler and faster. Therefore, RPA technology is promising for on-site detection and has the potential to be applied in a resource-limited setting.

Cytb gene is usually used in phylogenetic studies of mammalian species, and is one of the commonly used genetic loci in species identification, because *Cytb* exhibits little intra-specific variability and shows sufficient inter-specific variation (Mohamad, El Sheikha, Mustafa, & Mokhtar, 2013; Tobe, Kitchener, & Linacre, 2010). Therefore, the *Cytb* gene of duck was chosen as the target in this study, and the RPA primers and probes were designed targeting the sequences of *Cytb* gene. Furthermore, the real-time RPA and LFS RPA assays for rapid and sensitive detection of duck ingredient were developed and evaluated on different kinds of animal-derived foods.

2. Material and methods

2.1. Sample preparation and DNA extraction

The fresh meat samples (donkey, chicken, pig, goat, sheep, cattle, duck, Shelduck, Muscovy duck, Peking duck, and Cherry valley duck) were purchased from local supermarkets or collected in abattoirs. The above raw meats were churned and dried into a powder. Briefly, 500 g fresh meat was aseptically cut into thin slices and put into a blender with 500 mL deionized water, followed by intermittent stirring to puree and dried in the oven at 65 °C for 6 h. Then, the dried samples were flaked into a grinding jar of hybrid ball mill and ground into powders at 30 Hz/s for 2 min. Finally, these powders were sieved through different mesh according to their density separately. A 50 mg powder was used for nucleic acid extraction by Wizard® Genomic DNA Purification Kit (Promega, Madison, USA).

One hundred and thirteen meat and food samples were purchased from local supermarkets or submitted to our laboratory for testing between January 2021 and August 2022, which were different types and made by different processing methods. The classification of samples is provided in Table 1. All samples were taken 50 mg for nucleic acid extraction in two ways: i) conventional liquid nitrogen grinding combined with Wizard® Genomic DNA Purification Kit, named Method 1 in the following content; and ii) a rapid DNA extracted method combined the TIANcombi DNA Lyse&Det PCR Kit (TIANGEN, Beijing, China) with an electric mortar and pestle under the guidance of manufacturer's manual, named Method 2 in the following content.

Extracted DNA was examined for concentration and quality using an ND-2000c spectrophotometer (NanoDrop, Wilmington, USA) and stored at −20 °C until use.

2.2. Primers and probes of the RPA assays

The *Cytb* gene was selected as the target gene. The published nucleic acid sequences of ducks (*Anas platyrhynchos*, EU009397.1, EU755252.1, FJ167857.1, KJ689447, KJ739616.1, KJ833586.1, MF069249.1, MF069251.1, MH744426.1, MW354666.1, NC_009684.1), chicken (*Gallus gallus*, NC_053523.1), pig (*Sus scrofa*, NC_000845.1), goat (*Capra hircus*, NC_005044.2), donkey (*Equus asinus*, NC_001788.1), sheep (*Ovis aries*, NC_001941.1), and cattle (*Bos taurus*, NC_006853.1) were collected from GenBank. In this study, the location of primers/probes for real-time RPA and LFS RPA refer to the position in the duck Cytb gene of NC_009684.1, therefore, NC_009684.1 was analyzed with sequences of 10 other duck and 6 other species using MegAlign software (version 7.0; DNASTAR Inc., WI, United States). The analysis results showed that nucleotide sequence homology of duck Cytb gene within the duck were 99.8 % − 100 %, and homology with other species were 70.9 % − 83.0 %. The conserved regions among intra-species while highly mutable between inter-species were confirmed. Thereafter, 4 forward primers and 3 reverse primers for real-time RPA and additional 2 forward primers and 6 reverse primers functioned by biotin for LFS RPA were designed according to the RPA Assay Design Manual of TwistXD and synthesized by Generay (Shanghai, China). The exo probe for real-time

Table 1
Detection results of duck ingredient by real-time PCR in different kinds of foods.

Category	Title	Positive	Negative	Total
Duck-related	duck meat products under different thermal processed treatments (dried, roasted, marinated in sauce, minced meat, pressure canned, etc.)	8	0	8
Duck blood	–	3	1	4
Pig blood	–	1	2	3
Pork-related	stuffed pork and pork sausage	2	2	4
Lamb-related	raw/cooked shish kebabs and mutton slices, mutton meat, mutton sausage, etc.	10	45	55
Beef-related	raw/cooked beef skewers/slices, diced beef steak, luncheon meat, etc.	2	24	26
Other meat product	hand-torn meat strips, sausages, etc.	1	3	4
Quick-Frozen Food	wonton, dumplings and pies	3	6	9
		30	83	113

RPA and the nfo probe for LFS RPA were designed based on the amplified region of the optimal primer pairs. Besides the aforementioned primers and probes, the real-time PCR primers and TaqMan probe from the previously described protocol were also synthesized in this study (Liu et al., 2021). The related nucleotide sequences are shown in Table 2.

2.3. Reaction system

2.3.1. Real-time PCR

Real-time PCR was used to analyze 113 samples. The assay was performed in ABI Quant Studio 5 (Applied Biosystems, Foster City, California) using a solution containing 12.5 μL of 2 × PerfectStart® II Probe qPCR SuperMix (TransGen Biotech, Beijing, China), 2 μL of sample DNA, 1.0 μL of each primer and probe (10 μmol/L) and 7.5 μL of ddH_2O. The reaction condition was set as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s.

2.3.2. Real-time RPA

The real-time RPA reactions were performed following the manufacturer's recommended protocol of the ZC BioScience™ exo kit (ZC BioScience, Hangzhou, China). Briefly, 25 μL of A buffer, 2 μL of each primer (10 μmol/L), 0.6 μL of exo probe (10 μmol/L), 1 μL of control template or 2 μL of sample template, and ddH_2O were used to adjust the volume to 47.5 μL. Then, 2.5 μL of B buffer (magnesium acetate, 280 mmol/L) was added to the tube cap, followed by capping and inverted several times, and centrifuged instantaneously. The reaction tube was placed in the Genie III scanner device (OptiGene Limited, West Sussex, UK) at 39 °C for 20 min. The detection results were presented as threshold time (TT, mm:ss).

2.3.3. LFS RPA

The LFS RPA assays were performed using GenDx ERA Kit (GenDx Biotech, Suzhou, China) and reacted in the volume of 50 μL, which consists of 20 μL of rehydration buffer, 2.1 μL of each primer (10 μmol/L), 0.6 μL of nfo probe (10 μmol/L), 1 μL of control template or 2 μL of sample template, and ddH_2O were used to adjust the volume to 48 μL. Then, 2.0 μL of magnesium acetate (280 mmol/L) was added to tube cap. After the same operation as 2.3.2, the reaction tube was incubated in a metal bath. Finally, 5 μL of LFS RPA product was diluted 40-fold with ddH_2O, then the lateral flow strips (GenDx Biotech, Suzhou, China) were inserted into the diluent, and the results were visualized within 5 min.

2.4. Specificity and sensitivity of the RPA assays

The genomic DNA of 11 animal species were diluted to 1×10^5 pg/μL and the inter- and intra-species specificity was validated using RPA assays. Besides 1 μL duck genomic DNA as positive control and 1 μL ddH_2O as no template control, 1 μL genomic DNA of donkey, chicken, pig, goat, sheep, and cattle were used to test cross-reactivity. Meanwhile, 1 μL genomic DNA of Muscovy duck, Shelduck, Cherry valley duck, and Peking duck were used to verify the ability to identify different duck breeds. The specificity tests were conducted in triplicate to confirm the result.

Duck genomic DNA was 10-fold serially diluted from 1×10^5 to 1×10^0 pg/μL, and 1 μL was used as a template for RPA amplification to validate the sensitivity of the RPA assay. Furthermore, the adulteration sensitivity of RPA assays was also evaluated based on the presence of background DNA, and adulterated samples were prepared by mixing mutton powder with duck power in a series of proportions of 0.1, 0.5, 1, 5, 10, and 50 % (w/w). The sensitivity tests were conducted in triplicate to confirm the result.

2.5. Evaluation of the developed RPA assays on the samples and optimization of whole process

The DNA from 113 collected samples was extracted using Method 1 and Method 2, and the duck ingredient was detected using real-time PCR and the developed real-time RPA and LFS RPA with 2 μL extracted DNA as a template.

The procedure of the whole detection process is shown in Fig. 1.

3. Results and discussion

3.1. Screening of the optimal primers and probe combination

Previous studies had showed that mismatches scattering along both ends and the internal region of primer pairs simultaneously, especially near or at the 3′ end, could reduce amplification efficiency and even hamper RPA amplification reaction (Daher, Stewart, Boissinot, Boudreau, & Bergeron, 2015). Strategies for primer selection included searching heterogeneous gene regions among species and superimposing the 3′-end of primers on a mismatch, while the mismatch at 3′-end of primers were avoided among intra-species.

The basic RPA reaction was performed during the primer screening. $CytbF_{1\text{-}1}R_1$, $CytbF_{1\text{-}2}R_1$, $CytbF_2R_2$, and $CytbF_3R_3$ were verified using 1 μL of 1×10^5 pg/μL duck genomic DNA, and the target bands with expected

Table 2
Sequences of the primers and probes for duck real-time RPA, LFS RPA and real-time PCR assays.

Assays	Primers and probes	Sequence (5′~3′)	Location	Reference
Real-time RPA	$CytbF_{1\text{-}1}$	GATTCTCAGTGGATAACCCAACCCTAACCCG	503 ~ 533	This study
	$CytbF_{1\text{-}2}$	CCAACCCTAACCCGATTCTTCGCCATTCAC	520 ~ 549	
	$CytbR_1$	TAGGTGAGAATAGGGCTAGTGCTATGAGGG	716 ~ 745	
	$CytbF_2$	ACGCCATCCTGCGGTCAATCCCAAACAAACTA	836 ~ 867	
	$CytbR_2$	GAGTTGCCCGATGATGATGAATGGGTGTTCGA	1031 ~ 1062	
	$CytbF_3$	AATCGCAGGAATCACCCTAGTCCACTTAACC	567 ~ 597	
	$CytbR_3$	ATTCTGGTTTAATGTGTGGTGGGGTTACTAG	787 ~ 817	
	Cytb-exoP	TCGCAGGAATCACCCTAGTCCACTTAACCT[FAM][THF]C[*BHQ*1]ACACGAATCAGGCTC-C3-spacer	567 ~ 617	
RPA-LFS	$CytbF_{r2\text{-}1}$	TAACCCAACCCTAACCCGATTCTTCGCCATT	516 ~ 546	This study
	$CytbR_{r2\text{-}1}$	Biotin-AAGCATGAGGATAAATCCTAGGATGTCC	684 ~ 711	
	Cytb-nfoP	FAM-TCGCAGGAATCACCCTAGTCCACTTAACCTT[*THF*]CTACACGAATCAGGCTC-C3-spacer	567 ~ 617	
real-time PCR	GHF	GCCTCCACCCCTGATCCT	213 ~ 230	(Liu et al., 2021)
	GHR	CGCTCCCCACAGCTCTCA	302 ~ 319	
	GHP	(FAM)-TGCCCACACCCAAACCCACCAC-Eclipse	246 ~ 267	

Note: The location of primers/probes for real-time RPA and LFS RPA refer to the position in the *Cytb* gene of duck (Anas platyrhynchos, GenBank accession no. NC_009684.1); and the location of primers/probe for real-time PCR refers to the position in the *GH* gene of duck (Anas platyrhynchos, GenBank accession no. JN408702.2).

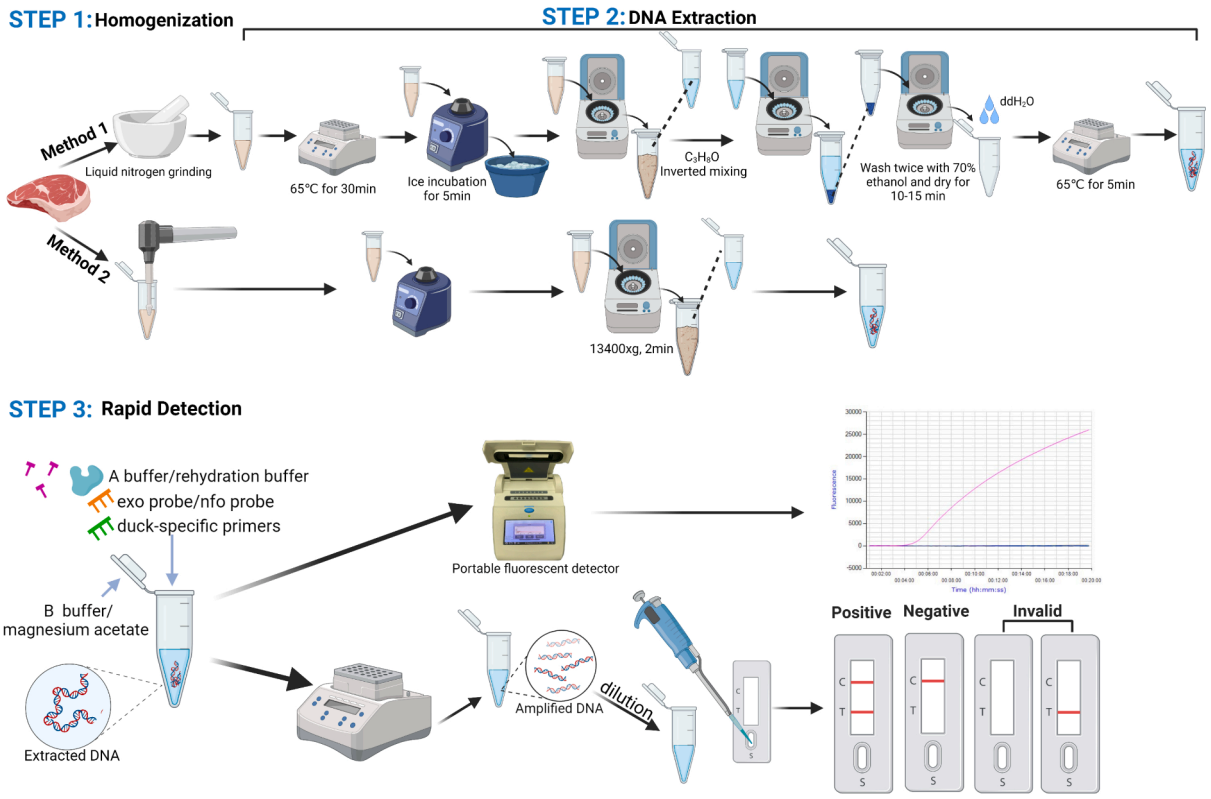

Fig. 1. Schematic diagram of the detection process for duck ingredient.

sizes appeared for each primer pair (243, 226, 227, and 251 bp) on 2.0 % agarose gel (Fig. S1A) after incubation at 39 °C for 20 min. Then, CytbF_1-$_2R_1$ was chosen as the optimal primer pair, and preliminary evaluation of the specificity was conducted using donkey, goat, chicken, cattle, sheep, and pig genomic DNA (Fig. S1B). The results showed that the primer pair was highly specific and produced an amplification band only for duck genomic DNA, while no cross-reactions with other common animal-derived ingredients were observed. The sensitivity validation resulted in a limit of detection (LOD) of 10^1 pg duck genomic DNA in a 50 μL reaction system, or 1×10^1 pg/reaction. Then the probes for real-time RPA and LFS RPA were designed based on the amplified Cytb$F_{1\text{-}2}R_1$ fragment. The most important step in designing Cytb-exoP for real-time RPA is to find a position within the target sequence where two adjacent thymine (T) residues are separated by 1 to 5 nucleotides. One T residue is at least 30 bases away from the 5′ end, while another is at least 15 bases away from the 3′ end. In Cytb-exoP, the two adjacent T residues were modified with a fluorophore and a quencher, respectively, while a base in the middle was replaced by tetrahydrofuran abasic–site mimic (THF), and a block added at the 3′ end prevents the oligonucleotide from acting as an amplification primer.

The sequences of real-time RPA primers and probes could be applied to LFS RPA with minor modifications. The 5′ end of the reverse primer was labeled with biotin, while the 5′ end of the probe was labeled with FAM. The THF site of the probe and 3′ end block remained unchanged. However, this study showed a false positive in LFS RPA using the same primers and probe sequence as real-time RPA. Therefore, two additional forward primers and six labeled reverse primers were designed near the Cytb-exoP position. After the cross-combination test with water as a template, it was determined that Cytb$F_{r2\text{-}1}R_{r2\text{-}1}$ did not show false positive results and thus served as the optimal primer pair for the subsequent tests (data not shown).

3.2. Specificity and sensitivity of real-time RPA assay

The results showed that the real-time RPA assay produced a specific amplification curve only for duck genomic DNA and no cross-reactions with other common animal ingredients, indicating a good inter-species specificity (Fig. 2 A). Meanwhile, duck ingredient was detected in different duck breeds, such as Muscovy duck, Shelduck, Cherry valley duck, and Peking duck, suggesting a certain degree of good intra-species specificity (Fig. 2 B). Results of three repeats were similar and supported the above conclusions.

Using 10-fold dilutions of duck genomic DNA and artificial mixed meat powder samples to determine the sensitivity of the real-time RPA assay. The results showed that duck genomic DNA down to 1×10^1 pg/reaction still produced significant amplification curve (Fig. 2 C); when the content of the duck ingredient was 0.1 %, the real-time RPA assay still showed a specific amplification curve (Fig. 2 D), indicating that the LOD of the real-time RPA assay was 0.1 %. The sensitivity experiments were performed three times and showed the same results.

For economic profit, the addition of poultry products in other meats corresponds to over 10 % of total meat weight (Liu et al., 2021). When adulteration is present, the level of sensitivity obtained is sufficient to cope with the actual demand in animal-derived ingredient detection. Some RPA-based methods for detection of animal-derived ingredient had been developed (Chen et al., 2022; Ivanov et al., 2021; Kumar et al., 2021). All of them achieved good detection limits or adulteration sensitivity, but none of them studied the effect of background DNA on the detection ability at different adulteration ratio. It had shown that background DNA reduced the detection ability of the method (Rohrman & Richards-Kortum, 2015), which resulted in the detection failure of the low doses of adulteration. Therefore, artificial samples mixed in different proportions of the target ingredient was used to evaluate the adulteration sensitivity of the developed RPA assays in this study. Compared to the same concentration without background DNA, the real-time RPA amplification efficiency showed negatively affected by 0.1 %

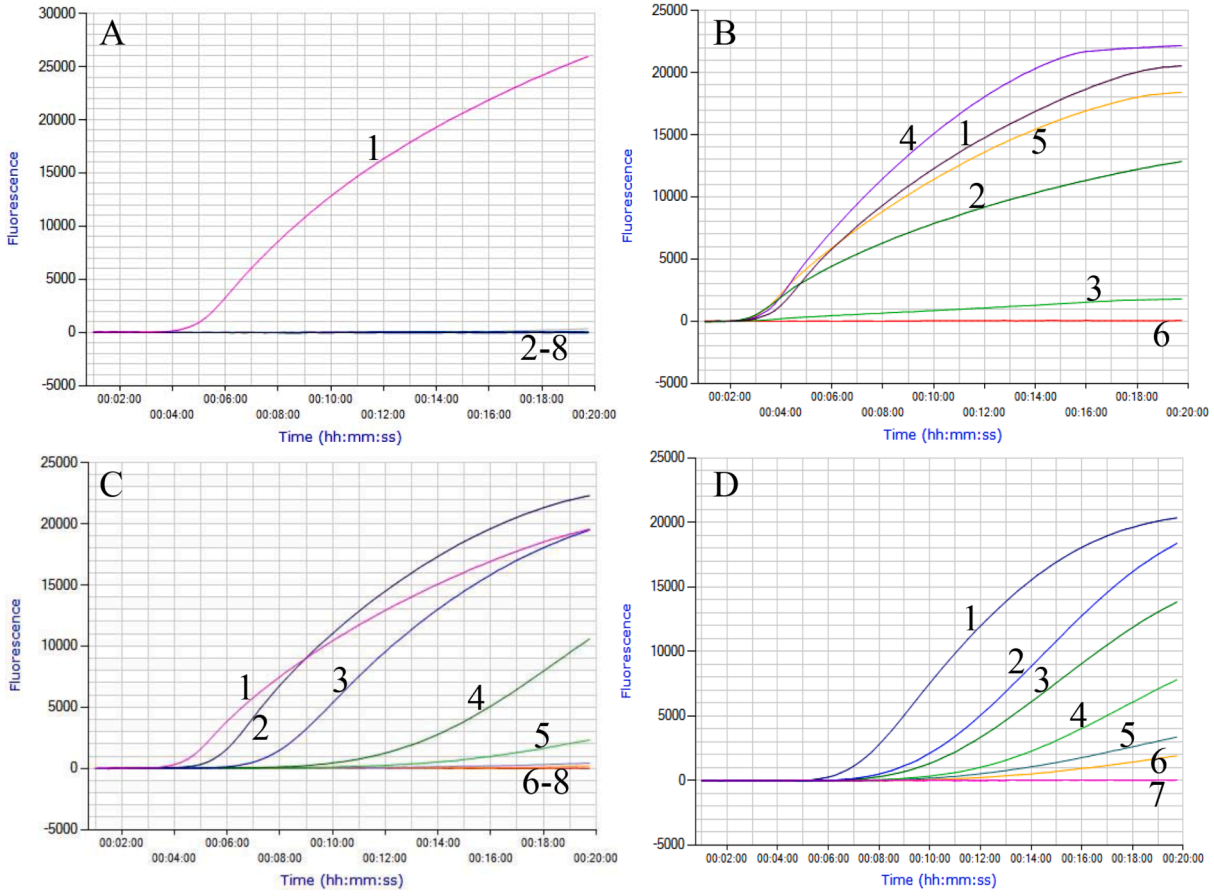

Fig. 2. Performance of the real-time RPA assay for duck ingredient. (A) Evaluation of the analytical inter-species specificity. Line 1, duck; line 2, donkey; line 3, sheep; line 4, goat; line 5, chicken; line 6, cattle; line 7, pig; line 8, ddH_2O. (B) Evaluation of the analytical intra-species specificity. Line 1, duck; line 2, Shelduck; line 3, Muscovy duck; line 4, Peking duck; line 5, Cherry valley duck; Line 6, ddH_2O. (C) Evaluation of the analytical sensitivity. Line 1, 1×10^5pg/reaction; line 2, 1×10^4 pg/reaction; line 3, 1×10^3pg/reaction; line 4, 1×10^2 pg/reaction; line 5, 1×10^1 pg/reaction; line 6, 1×10^0 pg/reaction; line 7, 1×10^{-1} pg/reaction; line 8, ddH_2O. (D) Evaluation of the adulteration sensitivity. Line 1, 50 % duck + 50 % sheep; line 2, 10 % duck + 90 % sheep; line 3, 5 % duck + 95 % sheep; line 4, 1 % duck + 99 % sheep; line 5, 0.5 % duck + 99.5 % sheep; line 6, 0.1 % duck + 99.9 % sheep; line 7, ddH_2O.

duck adulteration, which equivalent to approximately 100 pg/reaction (Ct = 30) of duck genomic DNA. We concluded that the presence of background DNA would affect the amplification reaction of RPA assays, resulting in the possibility of being undetected with below 100 pg/reaction at 0.1 % adulteration. However, this degree of adulteration cannot bring economic benefits and has no practical significance. Therefore, the background DNA effect cannot prevent the practical application of two RPA assays.

3.3. Optimization of incubation temperature and time of LFS RPA

We adjusted the reaction temperature and incubation time to improve the amplification efficiency and achieve the optimal reaction conditions of LFS RPA. Reaction temperature (35 ~ 43 °C) was optimized with 1 μL of 1×10^1 pg/μL duck genomic DNA as a template for 20 min of incubation. The results showed that the LFS RPA reaction worked successfully at 35 ~ 43 °C, and all test line were presented (Fig. S2 A). The test line was brighter at 40 °C compared to 39 °C, and there was no significant difference in band brightness between 40 and 43 °C, so the optimal temperature for the reaction was 40 °C. Furthermore, the effect of incubation time was also assessed at the optimum reaction temperature. The test line emerged after 10 min of incubation and became identifiable after 20 min of amplification (Fig. S2 B). The test line brightness increased as the reaction time increased, but there was no significant difference in the brightness from 20 to 30 min. Therefore, the optimal reaction time was determined to be 20 min.

3.4. Specificity and sensitivity of LFS RPA assay

The specificity of LFS RPA was confirmed at 40 °C for 20 min. The results showed that ducks including Muscovy duck, Shelduck, Cherry valley duck, Peking duck were detected with the test line emerged; and none of the other common animal ingredients, such as donkey, horse, chicken, cattle, sheep, and pig, were detected (Fig. 3 A), indicating a good inter- and intra-species specificity. The specificity validation experiments were conducted in triplicate and produced the similar results.

The sensitivity of the assay was confirmed using a dilution series of duck genomic DNA and different proportions of artificial mixed meat powder samples at 40 °C for 20 min. When the concentration of the template was 1×10^0 pg/μL, the test line still appeared despite being weak, while no test line appeared at 1×10^{-1} pg/μL, therefore, the sensitivity of the developed LFS RPA assay was 1×10^0 pg/reaction (Fig. 3 B). In terms of LOD, the test line could appear even if the amount of duck ingredient was as low as 0.1 %. Therefore, the LOD of the established LFS RPA assay was 0.1 % (Fig. 3 C). Similarly, the sensitivity validation experiments were conducted in triplicate and produced the similar results.

3.5. Evaluation of the developed RPA assays on the samples and optimization of whole process

The applicability of the developed real-time RPA and LFS RPA were evaluated by analyzing 113 different food samples, including duck meat

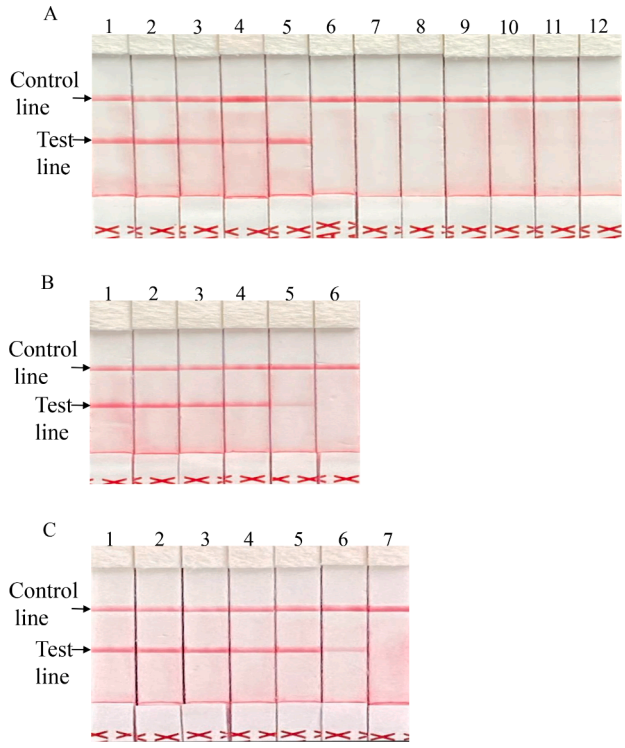

Fig. 3. Performance of the LFS RPA assay for duck ingredient. (A) Evaluation of the analytical intra-species and inter-species specificity. Lane 1, Shelduck; lane 2, Peking duck; lane 3, Cherry valley duck; lane 4, Muscovy duck; lane 5, duck; lane 6, donkey; lane 7, goat; lane 8, chicken; lane 9, cattle; lane 10, sheep; lane 11, pig; lane 12, ddH_2O. (B) Evaluation of the analytical sensitivity. Lane 1, 1×10^4pg/reaction; lane 2, 1×10^3pg/reaction; lane 3, 1×10^2pg/reaction; lane 4, 1×10^1pg/reaction; lane 5, 1×10^0pg/reaction; lane 6, 1×10^{-1}pg/reaction. (C) Evaluation of the adulteration sensitivity. Lane 1, 50 % duck + 50 % sheep; lane 2, 10 % duck + 90 % sheep; lane 3, 5 % duck + 95 % sheep; lane 4, 1 % duck + 99 % sheep; lane 5, 0.5 % duck + 99.5 % sheep; lane 6, 0.1 % duck + 99.9 % sheep; lane 7, ddH_2O.

products under different processing ways and blood products.

Firstly, the DNA from 113 samples was extracted by a conventional, time-consuming and reliable method named Method 1, and the duck ingredient was detected using real-time PCR, real-time RPA, and LFS RPA with 2 μL extracted DNA as a template. The results of real-time PCR in Table 1 showed that duck ingredients were detected in 30 samples (26.55 %, 30/113) and the classification of samples was also displayed in Table 1. After comparison the results between real-time PCR and developed RPA-based assays, the results showed that real-time PCR, real-time RPA, and LFS RPA produced identical qualitative results, and the cycle threshold (Ct) value of real-time PCR and TT value of real-time RPA corresponding to each positive sample were shown in Table 3. The data suggested that real-time RPA and LFS RPA assay could be well applicable to the actual samples with the comparable performance of real-time PCR. The DNA fragmentation owing to thermal processing affected the ability of DNA-based detection methods (Ali et al., 2012; Muhammed, Bindu, Jini, Prashanth, & Bhaskar, 2013; Sreenivasan Tantuan & Viljoen, 2021). Through testing duck meat products under different processing ways, such as being dried, roasted, marinated in sauce, minced meat, pressure canned, etc, the results demonstrated that the developed real-time RPA and LFS RPA assays were not affected by DNA fragmentation. It was better to evaluate the applicability of RPA assays using the actual samples than the simulated samples in a single processing procedure, because the actual food processing procedure usually was a combination of multiple processing ways.

Subsequently, in order to optimize the whole detection process for rapid detection of duck ingredient, we adopted the second approach, Method 2, to provide a simpler and faster way of DNA extraction through a grinding pestle with a battery for rapid homogenization. Similarly, the extracted DNA was also analyzed using real-time PCR, real-time RPA and LFS RPA. The final qualitative results using the DNA extracted in Method 2 remained consistent with those using the DNA extracted in Method 1 and a new round of Ct values of real-time PCR and TT values of real-time RPA corresponding to each positive sample are also shown in Table 3. The data suggested that rapid DNA extraction was a feasible step to combine with real-time RPA or LFS RPA. Overall, the rapid detection of duck ingredient was realized through the rapid sample homogenization, the crude nucleic acid extraction, and the rapid target gene amplification and results determination. The optimized whole process for duck ingredient detection performed well for the actual meat food samples, and it significantly reduced the whole detection process to less than 30 min. A previously described real-time PCR assay for duck ingredient detection (Liu et al., 2021) took approximately 2 h, and SN/T 3731.5–2013 is the current detection standard for duck ingredient in China, which requires PCR amplification combining with the subsequent sequencing and takes approximately 1–2 days.

It was noteworthy that the blood products were firstly applied as potentially adulterated food for the validation of method applicability. Duck blood is a common food in Chinese diet, which is tender and soft and usually used in hotpot. Because of its lower yield and higher cost, the duck blood is often replaced by pig blood. Relevant RPA methods had considered the effect of blood water on the detection of animal-derived ingredients (Kissenkotter et al., 2020), but the assay was not applied in the blood products. Through testing samples with more complex matrices, the developed RPA assays in this study were demonstrated to be successfully applied in duck ingredient detection in a wider range of animal-derived food. In this study, the TT values of real-time RPA corresponded to the Ct values of real-time PCR with an R^2 value of 0.8674 in meat samples (Fig.S4). Contrast to the fact that the complex meat matrices and ingredient could inhibit the PCR amplification efficiency, RPA could be applied to actual samples without extensive DNA purification processes (Rohrman & Richards-Kortum, 2015; Schrader, Schielke, Ellerbroek, & Johne, 2012). In this study, we found an interesting difference between the real-time PCR results and two RPA assays for the duck blood products with low Ct values. The results showed that whole blood affected the amplification reaction of real-time RPA and LFS RPA, and the extent of the effect depended on the nucleic acid extraction method. As for real-time RPA, the duck ingredient was not detected in the DNA extracted from pig blood product using Method 1, indicating that no false positive occurred, and an accumulation of fluorescence appeared for DNA extracted from duck blood products, but not the typical amplification curves (Fig.S5 A). For the extracted DNA by Method 2, the inhibition to RPA amplification was more pronounced in whole blood products, and the duck ingredient was barely detected in duck blood products (Fig.S5 C). Then the extracted DNA by Method 2 was subjected to LFS RPA, and no false-positive signals were produced. The ability to detect duck ingredient was seriously affected, displaying faint or absent bands at the location of the test line with duck blood products (Fig.S5 E). It was encouraging that a simple dilution process could significantly reduce the inhibitory effect. After a 10-fold dilution of extracted DNA with ddH_2O, duck ingredient was also undetected in pig blood product through real-time RPA and LFS RPA, while specific typical amplification curves and clear test lines were produced in real-time RPA and LFS RPA assays for the duck blood products (Fig. S5 B, D, and F).

4. Conclusion

The price of duck meat is several times lower than that of beef or lamb. If market regulation is not timely and effective, it is not conducive to maintain the fair trade. In this study, the developed real-time RPA and LFS RPA detected only the ducks and false positives were not observed for other common animal. The LODs of real-time RPA and LFS RPA were

Table 3
Detection of duck ingredient in different kinds of foods using the real-time RPA, LFS RPA, and real-time PCR combined with different DNA extracted methods.

Category	No.	Title	Method 1			Method 2		
			CT	TT(mm:ss)	LFS RPA	CT	TT(mm:ss)	LFS RPA
Duck-related	1	fried duck	9.37	3:30	+	11.24	3:00	+
	2	fried duck frame	18.25	7:00	+	11.23	3:00	+
	3	roasted duck	9.44	3:30	+	13.25	2:40	+
	4	roasted duck wing root	11.12	5:30	+	14.12	3:30	+
	5	roasted duck wing	19.04	7:00	+	14.25	3:30	+
	6	sauteed duck breast	16.33	5:00	+	11.36	3:00	+
	7	canned whole duck	22.92	8:00	+	25.41	9:30	+
	8	duck bacon	11	5:30	+	10.21	3:00	+
Duck blood (10x diluted)	34	duck blood #1	17.88	6:30	+	18.36	9:30	+
	35	duck blood #2	17.28	6:30	+	16.08	8:00	+
	36	duck blood #3	16.51	5:30	+	20.41	9:15	+
Pork-related	37	pork stuffing	25.12	8:00	+	25.78	9:00	+
	38	pork	27.20	9:00	+	25.15	9:30	+
Pig blood (10x diluted)	40	pig blood #2	17.45	6:30	+	20.39	9:15	+
Lamb-related	56	lamb roll	22.10	7:00	+	23.48	8:30	+
	57	lamb kebab	12.37	6:00	+	14.26	5:00	+
	58	lamb kebab	10.50	5:00	+	11.65	3:00	+
	59	lamb slice	21.24	7:30	+	22.36	8:00	+
	60	lamb kebab	13.20	6:00	+	13.37	4:00	+
	61	lamb kebab	21.22	7:30	+	22.75	8:00	+
	62	lamb kebab	14.11	5:00	+	15.68	4:00	+
	63	lamb kebab	10.14	5:00	+	13.38	3:30	+
	64	lamb kebab	22.17	7:00	+	23.55	8:00	+
	65	lamb kebab	15.14	5:30	+	15.45	4:00	+
Beef-related	19	canned beef luncheon meat	27.26	9:30	+	26.39	9:30	+
	20	diced beef (satay)	33.76	10:00	+	29.26	11:00	+
Other meat product	43	spicy grilled sausage	15.24	5:00	+	16.46	3:00	+
Quick-Frozen Food	105	small wonton (fresh pork)	21.51	6:30	+	23.62	5:30	+
	106	thin crust pie (black pepper beef flavor)	25.08	8:30	+	25.46	7:00	+
	107	thin crust pie (pork and scallion)	23.5	7:00	+	26.52	7:30	+

sufficient to test the actual samples. For 113 collected samples, the developed RPA assays could achieve the authentication of duck-derived ingredients in different meat and blood products. Combined with the optimized fast, user-friendly Method 2, it was less than 30 min from sample to answer without sophisticated equipment. Therefore, the developed duck-specific real-time RPA and LFS RPA assays demonstrated excellent potential for the rapid, simple, and reliable on-site detection of duck ingredient in heat-treated meat and blood products, which could provide technical support for combating meat fraud and safeguarding the rights of consumers.

CRediT authorship contribution statement

Cang Zhou: Methodology, Investigation, Writing – original draft. **Jinfeng Wang:** Methodology, Investigation. **Jialin Xiang:** Data curation. **Qi Fu:** Validation, Resources. **Xiaoxia Sun:** Validation, Resources. **Libing Liu:** Supervision, Project administration. **Lianfeng Ai:** Supervision, Project administration. **Jianchang Wang:** Conceptualization, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

This work was supported by the Science and Technology Program of Hebei province (21375501D) and the Key Science and Technology Winter Olympics Project (2020YFF0305004).

Ethical approval

Tissue samples for DNA extraction were collected from either slaughtered animals or animals presented for postmortem. As such no ethical approval was required in present study.

Data availability

All data generated or analyzed during this study are included in this published article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2023.100162.

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