

Closed-tube saltatory rolling circle amplification with hydroxynaphthol blue for visual on-site detection of peanut as an allergenic food

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ARTICLE INFO

Keywords:

Food allergy
Saltatory rolling circle amplification
Visual detection
Peanut

ABSTRACT

Peanut allergy as one of the major causes of food-induced anaphylaxis is usually life-threatening for specific individuals. Nowadays, the effective remedy is avoidance of consuming allergen-containing food, urging monitoring the presence of peanut in foods. Herein, we proposed a closed-tube saltatory rolling circle amplification (SRCA) assay with hydroxynaphthol blue (HNB) for visual on-site detection of peanut allergen, displaying highly specificity for Ara h 1-encoding gene. The results can be discriminated by naked eye without opening the tube, averting costly sophisticated apparatus and reducing the risk of cross-contamination. The assay was 1000-, 100- and 10-fold more sensitive than PCR, LAMP and SRCA, respectively. The relative limit of detection is 0.01% in a binary mixture. The relative sensitivity, specificity and accuracy of this method were 100%, 97.50% and 98.67%, respectively. Thus, the visual closed-tube HNB-SRCA assay with superiorities of easy-to-perform, cost-effective and user-friendly has excellent potential for on-site application of food allergy.

1. Introduction

Food allergy is considered a serious and increasing food safety and global public health issue, especially in industrialized countries (Alves et al., 2015; Montserrat et al., 2015). Eight most common allergens, including milk, egg, peanut, soybean, fish, shellfish, tree nuts and wheat, are found to trigger 90 % of food allergic reactions (Venkataratnam, Sarangapani, Cahill, & Ryan, 2019; Zhang, Wu, Wu, Ping, & Wu, 2019). Among these, peanut allergy is one of the leading causes of food-induced anaphylactic reactions (Jiang, Guo, Zhang, Sun, & Weng, 2021; Pollet et al., 2011). Even a trace amount of peanut can induce different allergic reactions, with the symptoms varying from mild rashes to potentially fatal anaphylactic shock (Su, Liang, & Tan, 2021; Weng & Neethirajan, 2016). Up to now, there is no effective therapy for peanut allergy, and strict avoidance of the consumption of peanut and peanut products is the most appropriate way to prevent peanut-allergic reactions (Jiang et al., 2021; Kim et al., 2021). Ara h 1, as one of the major peanut allergens, has a stable homo-trimer structure contributing to the hypersensitivity of the protein and is accountable for nearly 95 % of peanut-allergic

reactions (Kim et al., 2021; Tran, Knez, Janssen, Pollet, Spasic, & Lammertyn, 2013; Weng et al., 2016). Hence, Ara h 1 is considered a representative biomarker for peanut allergen detection.

Current analytical methods developed for peanut allergens mainly comprise immune-assay, mass-spectrometry (MS), and nucleic acid-based approaches. Immunoanalytical methods, including enzyme-linked immunosorbent assay (ELISA) (Jayasena, Koppelman, Nayak, Taylor, & Baumert, 2019; Montserrat et al., 2015), western blot (Guarino & Sciarillo, 2018) and lateral flow assay (Yin et al., 2019), are frequently used relying on specific antibodies for the detection of allergenic ingredients in food samples. However, antibodies are sometimes relatively challenging to produce with a limited shelf life and may become unstable under extreme experimental conditions (Shin, Reddy, Park, & Park, 2021; Tran et al., 2013). In recent years, MS has overcome the above limitations because the antibodies are not required. Notwithstanding, the MS is restricted to bulky and pricy instrumentation, complicated operation and specialized technicians (Sena-Torralba, Pallás-Tamarit, Morais, & Maquieira, 2020). Nucleic acid-based methods show several superiorities: (1) DNA possesses higher stability

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than proteins, which are usually confronted with denaturation under food processing. (2) DNA is also less affected by allergen extraction methods. (3) These techniques, such as polymerase chain reaction (PCR) (Pierboni et al., 2018; Zhang, Cai, Guan, & Chen, 2015), and loop-mediated isothermal amplification (LAMP) (Sheu, Tsou, Lien, & Lee, 2018), etc., have showed high specificity and sensitivity for the detection of Ara h 1-encoding gene from peanut allergen. It is worthwhile to remark that they have their disadvantages. PCR-based methods have some constraints, including complex thermal cycling procedures (requiring controlling three temperatures exactly) and specialized staffing, which induce inconvenience in on-site detection (Su et al., 2021). Furthermore, LAMP-based methods involve the requirement of multiple primers and carry-over contaminations, causing false-positive results (Momin et al., 2020). In this scenario, the development of simple, user-friendly and on-site detection methods for peanut allergen is an urgent need, especially for application in resource-limited or field situations.

Saltatory rolling circle amplification (SRCA), a well-established isothermal amplification method, was first reported by our team (Wang, Yang, Zhang, Meng, Ma, & Zhang, 2017), which is a simplified form of rolling circle amplification (RCA) assay (Lizardi, Huang, Zhu, Bray-Ward, Thomas, & Ward, 1998). Dependent on the template-independent properties of *Bst* DNA polymerase, DNA can be synthesized at the intersection of non-closed circular DNA. Subsequently, DNA is consecutively produced at the primary primer binding location, merely like saltating a gap. Afterwards, SRCA presents a similar amplification course to RCA. The amplification principle of SRCA is presented in Fig. S1. Unlike the RCA method, SRCA does not require ligase and padlock probes to realize the cyclization process, which is rapid, convenient and instrument-free. For the amplification of linear DNA, the total operation time of SRCA shortens at least 3 h than RCA, and the detection expense is about 20 % of that of RCA, exhibiting that the SRCA is more time-saving and inexpensive. Herein, combined with hydroxynaphthol blue (HNB) dye, a visual and straightforward HNB-SRCA detection assay for peanut allergen is first established. On the one hand, in the presence of HNB, there tends to be an obvious contrast in colour between positive and negative samples, which is user-friendly and suitable for on-site detection. On the other hand, the reaction is carried out in a single closed-tube at a constant temperature, with HNB added before amplification. Therefore, opening the tubes after amplification is averted and is beneficial for reducing the risk of cross-contamination.

As far as we know, this is the first time to combine HNB with SRCA technology for peanut detection as an allergenic food. In this study, we presented a closed-tube HNB-SRCA assay for visual on-site detection of encoding-gene of peanut allergen Ara h 1 in food. The specificity, absolute limit of detection, and relative limit of detection of the assay was evaluated. In addition, the detection of peanut allergen Ara h 1 in processed foods was conducted by comparison with PCR.

2. Materials and methods

2.1. Materials used

Twenty-three samples were composed of 5 peanut samples and 18 non-peanut samples. Five varieties of peanut samples, including Jihua 4, Jihua 13, Jinonghua 3, Yuhua 37, and Huayu 52, were collected from the North China Crop Planting Base (Hebei, China). Eighteen non-peanut samples, including plant-based and animal-based food allergens, were purchased from local markets: soybean, sesame, hazelnut, sunflower seed, walnut, chestnuts, almond, pistachio, maize, oats, canola, wheat, egg, milk, beef, chicken, fish and shrimp.

2.2. Primers design

The coding sequence of Ara h 1 (No. AF432231, 2674 bp) was from

GenBank. The primers were designed using Primer Premier 5.0 and DNAMAN, respectively. All primers were synthesized by Takara Biotechnology Co., Ltd. (Dalian, China) and presented in Table 1.

2.3. DNA extraction and specificity assay

Genomic DNA was extracted from 100 mg of each sample using the High Efficiency Plant Tissue Genomic DNA Extraction Kit (DP350) (Tiangen Biotech Co., Ltd, Beijing, China) according to the manufacturer's instructions. The quantity and quality of the DNA was determined using NanoDrop 2000 UV-vis spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, Delaware, USA).

The specificity assays of HNB-SRCA and SRCA were performed using all samples listed in Table 2. Peanuts (Five varieties: Jihua 4, Jihua 13, Jinonghua 3, Yuhua 37, and Huayu 52) were used in all reactions as positive controls. Each test was repeated three times.

2.4. Closed-tube HNB-SRCA, conventional SRCA, LAMP and PCR reaction conditions

The closed-tube HNB-SRCA was performed in a total volume of 20 μ L: dNTPs (5 μ L, final concentration of 0.625 mM), $MgSO_4$ (3 μ L, final concentration of 3 mM), 10 $\times$ ThermoPol Buffer (2.5 μ L), each primer (1 μ L, final concentration of 0.5 μ M), DNA template (1 μ L, 62.5 ng/ μ L), *Bst* DNA polymerase (1.5 μ L, final concentration of 0.6 U/ μ L), HNB (0.8 μ L, final concentration of 240 μ M) and RNase-free water. First, an initial denaturation was 94 $^{\circ}C$ for 3 min and on ice for 2 min. Amplification was carried out at 62 $^{\circ}C$ for 45 min, followed by 80 $^{\circ}C$ for 5 min to end the reaction. All the reactions were fulfilled in a simple water bath. The other reaction conditions of the conventional SRCA assay (without HNB) were the same as the closed-tube HNB-SRCA.

The LAMP reaction was conducted in a reaction mixture of 25 μ L including 10 $\times$ ThermoPol Buffer (2.5 μ L), 8 U of *Bst* DNA polymerase (1 μ L), 0.2 μ M outer primers (F3 and B3 primers each), 1.6 μ M inner primers (FIP and BIP primers each), DNA template (1 μ L, 62.5 ng/ μ L), 1.2 mM $MgSO_4$, 350 μ M dNTPs and RNase-free water. The mixtures were reacted at 61 $^{\circ}C$ for 60 min followed by 80 $^{\circ}C$ for 5 min to end the reaction.

The PCR was accomplished in a volume of 25 μ L: 2 $\times$ Taq PCR MasterMix (11 μ L), each primer (1 μ L), DNA template (1 μ L, 62.5 ng/ μ L) and RNase-free water. The reaction was carried out as follows: denaturation at 94 $^{\circ}C$ (5 min), 30 cycles of 94 $^{\circ}C$ (1 min), 55 $^{\circ}C$ (45 s), and 72 $^{\circ}C$ (45 s); elongation at 72 $^{\circ}C$ (45 s).

The electrophoresis experiment of conventional SRCA, LAMP and PCR assays was performed using 1.6 % agarose. All assays were completed independently in triplicates. The DNA templates were replaced by RNase-free water in negative controls.

2.5. Analysis of the absolute and relative limit of detection of the closed-tube HNB-SRCA assay

To evaluate the absolute limit of detection of the closed-tube HNB-SRCA, 10-fold serial dilutions were prepared from extracted genomic DNA of peanut (Jihua 4) at the initial concentration of 62.5 ng/ μ L. The relative limit of detection of the closed-tube HNB-SRCA was determined in artificially mixed samples, including peanut powder and soybean powder. Peanut powder with different proportional gradients (100 %, 5 %, 2 %, 1 %, 0.5 %, 0.1 %, 0.05 %, 0.01 % and 0.005 % (w/w)) was added to soybean powder to prepare the binary mixtures for subsequent DNA extraction. In order to verify the credibility and repeatability of the results, each assay was performed in ten times.

The absolute and relative limits of detection were also confirmed using the conventional SRCA, LAMP and PCR assays, respectively. Each assay was performed in triplicate.

Table 1
Primers used in this study.

Method	Sequences	Length (bp)	Target fragment (bp)	Position in the target sequence (length, 2674 bp)
SRCA/ PCR	FWP: 5'AAGAAAGGCTCCGAAGAAGA3'	20	77	1637–1656
	RVP: 5'TTGTTAGAAAGATCGGGGCTC3'	20		1694–1713
LAMP	F3: 5'GGGAGGGTTTCTCCACTGA3'	19	42	97–115
	B3: 5'AGTGTGCCTCGAGGATCA3'	19		309–327 (B3c) ^a
	FIP: 5'GCGCAGGGTTCTCTGTTTCT(F1c)-ATCCTTGTCTGGCTTCAGT(F2)	42		185–206 (F1) ^b
	3'			130–149 (F2)
	BIP: 5'TGCCTCCAGAGTTGTCAACAGG(B1c)-CATACTCGAGCTTGGTGCA(B2)	41		214–235 (B1c)
	3'			274–292 (B2c) ^c

^a B3c sequence is the reverse complementary sequence of B3 sequence.

^b F1 sequence is the reverse complementary sequence of F1c sequence.

^c B2c sequence is the reverse complementary sequence of B2 sequence.

Table 2
Specificity results of the closed-tube HNB-SRCA developed in this study.

No.	Common Name	Scientific name	HNB-SRCA results	SRCA results
1	Peanut	<i>Arachis hypogaea</i> (Jihua 4)	+	+
2	Peanut	<i>Arachis hypogaea</i> (Jihua 13)	+	+
3	Peanut	<i>Arachis hypogaea</i> (Jinonghua 3)	+	+
4	Peanut	<i>Arachis hypogaea</i> (Yuhua 37)	+	+
5	Peanut	<i>Arachis hypogaea</i> (Huayu 52)	+	+
6	Soybean	<i>Glycine max</i>	–	–
7	Sesame	<i>Sesamum indicum</i>	–	–
8	Hazelnut	<i>Corylus heterophylla</i>	–	–
9	Sunflower seed	<i>Helianthus annuus</i>	–	–
10	Walnut	<i>Juglans regia</i>	–	–
11	Chestnuts	<i>Castanea mollissima</i>	–	–
12	Almond	<i>Amygdalus communis</i>	–	–
13	Pistachio	<i>Pistacia vera</i>	–	–
14	Maize	<i>Zea mays</i>	–	–
15	Oats	<i>Avena sativa</i>	–	–
16	Canola	<i>Brassica campestris</i>	–	–
17	Wheat	<i>Triticum aestivum</i>	–	–
18	Egg	<i>Gallus gallus</i>	–	–
19	Milk	<i>Chinese Holstein cows</i>	–	–
20	Beef	<i>Bos taurus</i>	–	–
21	Chicken	<i>Gallus gallus</i>	–	–
22	Fish	<i>Oreochromis mossambicus</i>	–	–
23	Shrimp	<i>Palinuridae</i>	–	–

2.6. Application of the closed-tube HNB-SRCA assay for the detection of processed food

Seventy-five commercial processed food products from different brands with different label declarations were purchased and detected by traditional PCR, LAMP and the closed-tube HNB-SRCA method, respectively. The food products are composed of 2 unpackaged peanuts, 1 unpackaged boiled peanut, 5 packaged peanuts, 3 fried peanuts with butter, 4 coated peanuts, 15 biscuits, 12 cakes, 10 chocolates, 7 edible oil, 6 beverages, and 10 other foods. The detailed information is presented in Table S1.

In this study, the traditional PCR is defined as the reference method. The relative sensitivity, specificity, and accuracy of the closed-tube HNB-SRCA method were determined by comparison with the experimental results of traditional PCR. The relative sensitivity, specificity, and accuracy were calculated with the following equations (ISO, 2003; Zhang, Yang, Li, Yuan, & Zhang, 2019):

$$\text{Relative sensitivity: } (T_{\text{pos}})/(T_{\text{pos}} + F_{\text{neg}}) \times 100 \%$$

$$\text{Relative specificity: } (T_{\text{neg}})/(T_{\text{neg}} + F_{\text{pos}}) \times 100 \%$$

$$\text{Relative accuracy: } (T_{\text{pos}} + T_{\text{neg}})/(T_{\text{pos}} + T_{\text{neg}} + F_{\text{pos}} + F_{\text{neg}}) \times 100 \%$$

where T_{pos} is the true positive result by traditional PCR, LAMP and closed-tube HNB-SRCA method, T_{neg} is considered the true negative result by traditional PCR, LAMP and closed-tube HNB-SRCA method, F_{pos} is negative result by traditional PCR method and positive result by LAMP or closed-tube HNB-SRCA method, F_{neg} represents positive result by traditional PCR method and negative result by LAMP or closed-tube HNB-SRCA method.

3. Results and discussion

3.1. Overview of the detection procedures

In this study, we present a closed-tube HNB-SRCA assay for visible detection of encoding DNA region of Ara h 1 in foods, thus providing a rapid, convenient, closed-tube and sophisticated-equipment free method for on-site application. The schematic for the detection procedure of Ara h 1 is presented in Fig. 1. First, the DNA of peanuts can be easily extracted using the High Efficiency Plant Tissue Genomic DNA Extraction Kit. After simple extraction, the solution containing the target region encoding Ara h 1 is amplified by SRCA. The visual detection of SRCA amplicons by the naked eye was realized using HNB without opening the tubes, thus avoiding cross-contamination. Once the isothermal reaction starts, the pyrophosphate is simultaneously formed by adding dNTPs into the new-borne DNA strand. As the reaction continues, the quantity of pyrophosphate increases substantially, thereby producing a stable compound with magnesium. Hence, the concentration of free magnesium ions is reduced. Therefore, the colour of HNB varies from violet to sky blue (Daskou et al., 2021).

SRCA, as a novel isothermal approach established by our team, is a similar and simplified form of the RCA initially reported in Nature genetics by Lizardi et al. (Lizardi et al., 1998). Due to the easy operation, speed, specificity, high sensitivity and cost-effectiveness, SRCA has been increasingly utilized as a powerful detection tool for various food-borne pathogens (Wang et al., 2017; Yang, Zhang, Li, Lu, Yuan, & Zhang, 2019; Zhang, Yang, Li, Yuan, & Zhang, 2019) and meat adulteration (Hu, Xu, Zhang, Lu, Yang, & Zhang, 2021). HNB, as a low-cost and commercially available indicator dye, gained increased popularity in the monitoring of amplification results in LAMP (Fu et al., 2020; Pang et al., 2019). SRCA and LAMP as isothermal amplification methods can be conducted in a relatively simple and cheap heating device such as a water bath, which obviates the necessity for costly and complex thermocycler. Compared with LAMP, only one pair of primers is adequate for SRCA to amplify the targets, thus reducing the detection cost (approximately 50 % of the expenditure of LAMP) and the possibility of interaction among the primers (Yang et al., 2019). In most isothermal amplification reactions, aerosols might form in the tube. Once the tube lid is opened for subsequent electrophoresis or the addition of fluorescent dyes after reaction,

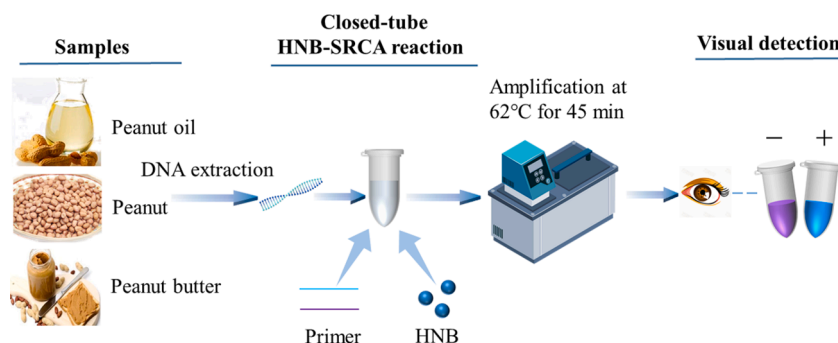


Fig. 1. Schematic of overall detection procedures.

cross-contamination might occur (Chen, Wang, Liu, Qi, & Guo, 2021). In the present study, all components required in SRCA and HNB were added to the centrifuge tube before the reaction started forming a closed-tube state, thereby eliminating the possible contamination and enhancing the detection accuracy.

3.2. Verification of the closed-tube HNB-SRCA products

As shown in Fig. S2A, on the visualized closed-tube HNB-SRCA platform, the positive result with a sky blue colour could be differentiated from the negative control with violet colour by the naked eye. By agarose gel electrophoresis experiment, the positive control presented a ladder-like DNA banding pattern, whereas no amplification was observed in the negative control (Fig. S2B). Therefore, clear visualization of the closed-tube HNB-SRCA amplicons by the naked eye is realized and suitable for less-advanced laboratories.

To validate the SRCA products of Ara h 1-encoding gene, sequence analysis of four bands numbered 1–4 in Fig. S2B was performed. As shown in Fig. S2C, the amplification products presented 100 % identity to the Ara h 1-encoding gene, which were composed of tandem-repeated units including the target sequence and the nucleotides added between the 3' end and the 5' end of the template. The results exhibited that the Ara h 1-encoding gene could be specifically amplified by SRCA.

3.3. Specificity of the detection method

As we all know, Ara h 1 constitutes the highest portion of peanut total protein and plays a critical role in peanut allergenicity (Gezer, Liu, & Kokini, 2016). Ara h 1, exists in the form of a homotrimeric compound, which is still stable even after heating, high pressure and hydrolysis during the food process (He et al., 2020). Hence, Ara h 1 is considered an ideal biomarker and possesses absolute priority in many reported detection methods for peanut allergen, thereby guaranteeing

the compliance of food labelling (Ji et al., 2011; Singh, Sharma, Baltus, & Suni, 2010; Sun et al., 2015).

In the present study, the specificity assay was determined using 5 peanut samples and 18 non-peanut samples targeting Ara h 1-encoding gene (shown in Table 2). As shown in Fig. 2, five peanut samples displayed the sky blue colour (Fig. 2A) and the typical ladder-like bands (Fig. 2B), suggesting positive results. However, the visual outcomes and electrophoresis images were negative for 18 non-peanut samples. In all, the closed-tube HNB-SRCA assay for detection of peanut targeting the Ara h 1-encoding gene revealed excellent specificity.

3.4. Absolute limit of detection of the visual and closed-tube HNB-SRCA assay

Templates extracted from peanut (Jihua 4) with concentrations ranging from 6.25×10^7 fg/ μ L to 6.25×10^{-1} fg/ μ L were applied to confirm the absolute limit of detection of the closed-tube HNB-SRCA assay, simultaneously with conventional SRCA, LAMP and PCR for comparison. Under 10 times of independent tests, the absolute limits of detection for the visual and closed-tube HNB-SRCA assay (Fig. 3A and Fig. S3) were found to be 6.25×10^0 fg/ μ L of peanut DNA, which is 10 times sensitive than conventional SRCA (6.25×10^1 fg/ μ L, Fig. 3B), 100 times sensitive than LAMP (6.25×10^2 fg/ μ L, Fig. 3C), and 1000 times more sensitive than PCR (6.25×10^3 fg/ μ L, Fig. 3D), respectively.

3.5. Relative limit of detection of the visual and closed-tube HNB-SRCA assay

To investigate the relative limit of detection of the closed-tube HNB-SRCA, the binary mixtures containing peanut powder and soybean powder with different proportional gradients were prepared. After 10 times of independent tests, the visual and closed-tube HNB-SRCA assay (Fig. 4A and Fig. S4) can detect as low as 0.01 %, which was 5-fold

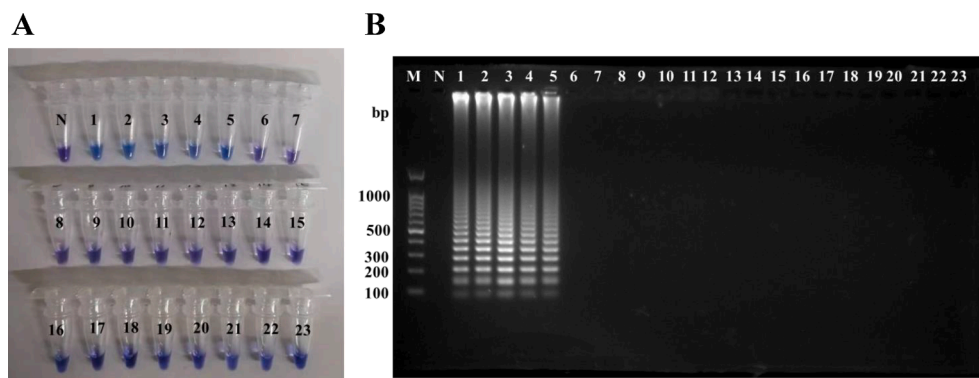


Fig. 2. Specificity results of the detection assay. (A) Visual results by the naked eye; (B) Amplification results by 1.6 % agarose gel electrophoresis experiment. M–100 bp DNA ladder; N–negative control; 1–23, corresponding to No.1 to No.23 listed in the Table 2.

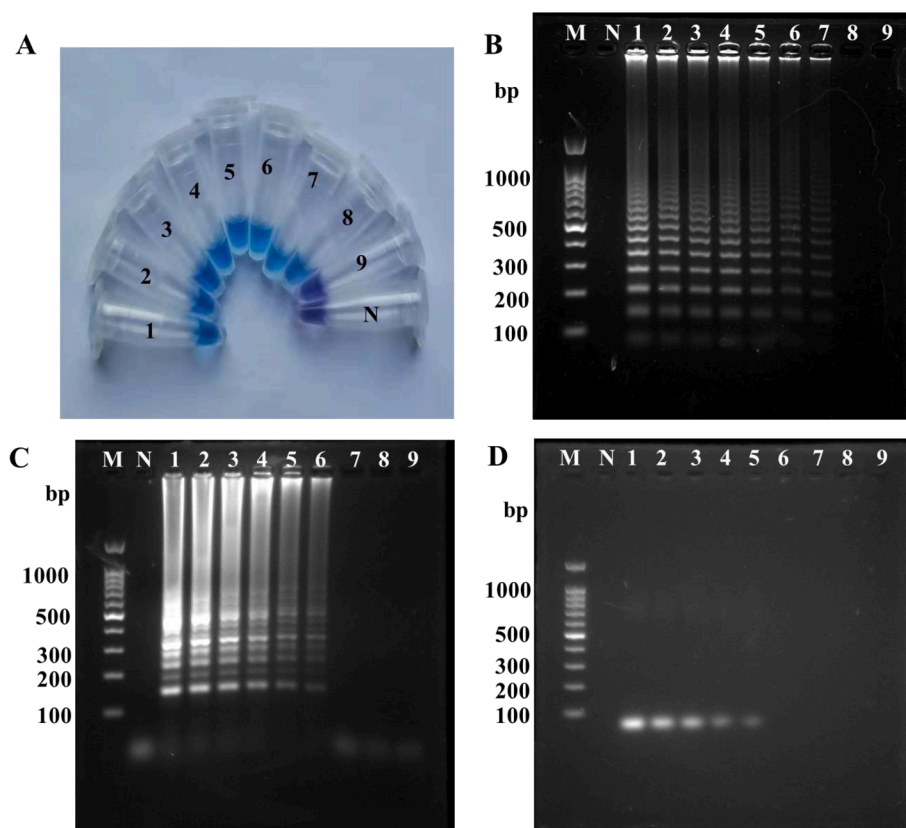


Fig. 3. Absolute limits of detection of the visual and closed-tube HNB-SRCA (A), conventional SRCA (B), LAMP (C) and PCR (D) for Ara h 1-encoding gene. M–100 bp DNA ladder; N–negative control; 1–9: 6.25×10^7 fg/ μ L– 6.25×10^{-1} fg/ μ L. All the electrophoresis experiment was performed using 1.6 % agarose.

better than that of conventional SRCA (0.05 %, Fig. 4B) and LAMP (0.05 %, Fig. 4C) and 50-fold better than that of PCR (0.5 %, Fig. 4D), respectively.

Nowadays, the detection methods for peanut allergy mainly include allergenic protein-based methods and nucleic acid (encoding an allergenic protein)-based methods. ELISA is the most frequently utilized protein-based method for monitoring allergens by both food industries and official food regulatory authorities. As reported by Montserrat et al. (2015), direct competitive and sandwich ELISA approaches were established to detect Ara h 1 in model biscuits containing commercial peanut butter, respectively. The sandwich assay based on Ara h 1 protein could not detect biscuits samples containing 5.0 % peanut addition. While, the direct competitive format for Ara h 1 proteins could detect samples containing 1.0 % peanut. Ji et al. (2011) developed a gold immunochromatography assay (GICA) using two monoclonal antibodies (mAb) against the peanut allergen Ara h 1, which was specific for peanut samples with a sensitivity of 10 ng/mL. The above mentioned immunoassay methods, dependent on using high affinity antibodies, possesses the merits of detecting specific allergen proteins in complicated mixtures. However, the allergen proteins are vulnerable to being damaged under harsh food processing methods such as high pressure, thermal and pulsed treatment, thereby affecting the detection accuracy (Johnson et al., 2010). Otherwise, the results may be misled by cross-reactivity with homologous proteins (Sena-Torralba et al., 2020). Alternatively, near-infrared (NIR) hyperspectral imaging (HIS) is a promising technique for offering non-destructive detection of diverse food samples, which is gradually applied for food allergen detection. Peanut flour in wheat flour (Laborde et al., 2020) and chocolate powder (Laborde, Puig-Castellví, Jouan-Rimbaud Bouveresse, Eveleigh, Cordella, & Jaillais, 2021) was detected using NIR-HIS, respectively. The results indicated that peanut adulteration could be detected in chocolate powder and wheat flour with the lowest concentration level of 0.1 % and 0.2 % (w/

w), respectively, which is similar to the relative limit of detection of traditional PCR (0.5 %) in our study. The NIS-HIS provides the rapid and non-destructive screening of the chemical heterogeneity of the samples, avoiding preparing nor altering the samples. However, this technology is hindered by the demand for pricy and cumbersome apparatus.

Nucleic acid-based methods contain PCR-based assays and isothermal amplification methods such as LAMP, RCA and SRCA. SRCA, as an emerging isothermal amplification approach established by our research team, is a combination of PCR (entailing only one pair of simple primer like PCR) and isothermal amplification (without thermal cycling procedures). The primer and reaction system of SRCA (requiring a single enzyme named *Bst* DNA polymerase) is simple. Otherwise, SRCA averts the necessity of padlock probes and ligase to accomplish the cyclization process. Hence, the detection time is saved, and the detection expenditure is reduced. While, conventional SRCA relies on agarose gel electrophoresis to judge the results. Stephan and Vieths (2004) developed a real-time PCR method for the peanut-specific DNA detection, with the limit of detection lower than 0.001 % (<10 ppm, w/w) peanut in processed foods. Sanchiz et al. (2021) applied chloroplast markers for specific detection of peanut by real-time PCR, and its limit of detection was 0.001 % (10 mg/kg, w/w) in binary mixtures of processed samples. Zhang et al. (2015) reported a real-time PCR method for detecting DNA targeting the allergenic protein Ara h 1 with a limit of detection of 0.005 % (w/w). Although above real-time PCR methods achieved lower limits of detection than that of our close-tube HNB-SRCA assay, it is worth mentioning that these real-time PCR methods always depend on the expensive and bulky real-time PCR instruments. Particularly, our close-tube HNB-SRCA assay with the advantages of equipment-free and visual on-site detection provided potential possibilities as a cheaper alternative to real-time PCR methods.

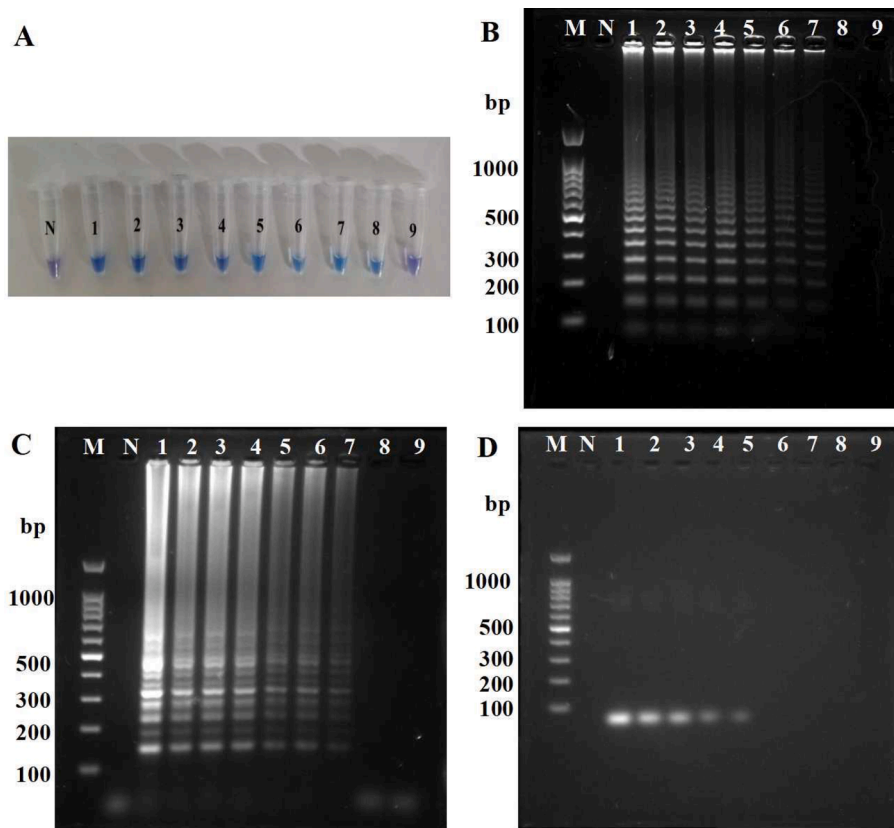


Fig. 4. Relative limits of detection of the visual and closed-tube HNB-SRCA (A), conventional SRCA (B), LAMP (C) and PCR (D) for Ara h 1-encoding gene. M–100 bp DNA ladder; N–negative control; 1–100 %; 2–5 %; 3–2 %; 4–1 %; 5–0.5 %; 6–0.1 %; 7–0.05 %; 8–0.01 %; 9–0.005 % (w/w). All the electrophoresis experiment was performed using 1.6 % agarose.

3.6. Applicability of visual and closed-tube HNB-SRCA for the detection of processed foods

To evaluate its potential for practical applications, 75 commercial food products with different label declarations were analyzed by using the traditional PCR, LAMP and closed-tube HNB-SRCA. 31 products contain peanut as the main ingredients or one of the ingredients, 22 products with trace amount of peanut, 10 sharing production line with peanut products and 12 were unlabelled. The detection results were presented in **Table S1**. 35 samples were detected as positive results by traditional PCR (reference method) and LAMP. However, 36 processed food samples were verified to contain peanut composition (positive results) by closed-tube HNB-SRCA in 75 samples. Therefore, the number of T_{pos} is 35 and the number of F_{pos} is 1. The left 40 samples were detected as negative results by traditional PCR and LAMP, but 39 samples were detected as negative results by closed-tube HNB-SRCA. Therefore, the number of T_{neg} is 39. As there is no sample presenting positive result by traditional PCR method and negative result by LAMP or closed-tube HNB-SRCA method, the number of F_{neg} is 0. Compared with the traditional PCR, the relative sensitivity, relative specificity, and relative accuracy of the visual and closed-tube HNB-SRCA method was calculated as 100 %, 97.50 % and 98.67 %, respectively (shown in **Table S2**).

4. Conclusions

In this work, we have utilized the remarkable superiority of HNB in that HNB is mixed before amplification compared with other color-based assays. Therefore, the necessity of opening the reaction tube to add the dye is averted, thereby decreasing the risk of cross-contamination. In summary, we demonstrated a closed-tube HNB-SRCA for visual and on-site detection of Ara h 1-encoding gene from

peanut in processed foods. The major advantage of HNB-SRCA over conventional SRCA is the closed-tube, simplicity, time-saving, sophisticated electrophoresis-free and inexpensiveness. In combination with HNB, the amplification products can be straightforwardly detected by the naked eye, omitting complicated agarose gel electrophoresis, which is indispensable in conventional SRCA. The absolute limit of detection of HNB-SRCA assay was 6.25×10^0 fg/ μ L, which was 1000 times more sensitive than PCR, 100 times sensitive than LAMP, and 10 times sensitive than conventional SRCA, respectively. Additionally, the peanut can be detected with the lowest level of 0.01 % in the binary mixture containing peanut powder and soybean powder. Due to the high specificity, sensitivity, and cost-effectiveness, the closed-tube HNB-SRCA assay holds a promising potential. It provides an ideal routine screening method for the on-site detection of food allergens, especially appropriate for resource-poor settings for surveillance of food allergen labelling of food products.

CRediT authorship contribution statement

Qian Yang: Conceptualization, Writing – original draft, Methodology, Validation, Funding acquisition. **Haoyu Yang:** Writing – review & editing, Software, Data curation. **Ning Yuan:** Methodology, Formal analysis, Investigation. **Shuna Zuo:** Methodology, Software, Validation. **Yunzhe Zhang:** Data curation, Resources. **Wei Zhang:** Conceptualization, Funding acquisition, Project administration, Supervision.

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.

Acknowledgements

The authors are grateful to the financial support from the National Natural Science Foundation of China (32172288 and 31371772), the Natural Science Foundation of Hebei Province (C2019204342), the Central Government Guided Local Science and Technology Development Fund Projects (Basic Research Projects) (216Z5501G), the Scientific Research Program of Hebei Education Department (QN2022073), the Advanced Talents Incubation Program of the Hebei University (521100222024), the Talent Introduction Project of Hebei Province (360-0803-JSN-3YGS) and the Food Processing Discipline Group of Hebei Agricultural University (No. 2021-06).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2022.133408>.

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