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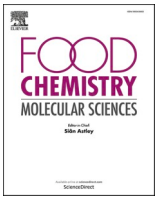

Establishment of rapid extraction and sensitive detection system of trace corn syrup DNA in honey

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ABSTRACT

Honey adulteration with exogenous syrup has become a common phenomenon, and current detection techniques that require large instruments are cumbersome and time-consuming. In this study, a simple and efficient method was developed by integrating the rapid extraction of nucleic acids (REMD) and recombinase polymerase amplification (RPA), known as REMD-RPA, for the rapid screening of syrup adulteration in honey. First, a rapid extraction method was developed to rapidly extract corn syrup DNA in five minutes to meet the requirements of PCR and RPA assays. Then, the RPA method for detecting endogenous maize genes *(ZssIIb)* was established, which could detect 12 copies/μL of the endogenous maize gene within 30 min without cross-reacting with other plant-derived genes. This indicated that the RPA technique exhibited high sensitivity and specificity. Finally, the REMD-RPA detection platform was used to detect different concentrations of corn syrup adulteration, and 1 % adulteration could be detected within 30 min. The 22 commercially available samples were tested to validate the efficacy of this method, and the established RPA was able to detect seven adulterated samples in less than 30 min. Overall, the developed method is rapid, sensitive, and specific, providing technical support for the rapid field detection of honey adulteration and can serve as a reference for developing other field test methods.

1. Introduction

Honey is a natural sweet substance produced by bees through the collection of nectar, secretions, or honeydew from plants (Codex, 1995). It is valued for its unique flavor, high nutritional content, and potential therapeutic properties, and its market demand is increasing. Recently, the problem of honey adulteration has become more serious. Typically, honey is adulterated with inexpensive sweeteners like sugar syrup, which has a similar fructose-to-glucose ratio to honey (Siddiqui, Musharraf, Choudhary, & Atta ur, 2017). Corn syrup is one of the most commonly used adulterants and is often used to feed bees or adulterate honey, affecting not only consumer health but also beekeepers. Therefore, the detection of honey adulteration has attracted widespread attention.

Currently, various methods, including chemical composition- and DNA-based analyses, have been used to detect syrup adulteration in honey. Chemical composition-based methods include thin-layer chromatography (Puscas, Hosu, & Cimpoiu, 2013), stable carbon isotopic ratio analysis (Çinar, Ekşi, & Coşkun, 2014), Raman spectroscopy (Li, Shan, Zhu, Zhang, & Ling, 2012), infrared spectroscopy (Chen, Xue, Ye, Zhou, Chen, & Zhao, 2011), and nuclear magnetic resonance (Li et al., 2017). However, these methods are usually time-consuming and require large instruments. Stable carbon isotopic ratio analysis can only detect molasses produced by C_4 plants but not C_3 plants. Thin-layer chromatography has limited application due to the requirement for hydrolysis of oligosaccharides and polysaccharides, and it is prone to false positives due to the presence of small oligosaccharides in pure honey. Although it is time-saving, Raman spectroscopy requires specialized personnel to analyze the samples, limiting its application (Du et al., 2015; Zhang, Gu, Liu, Qing, & Nie, 2023). Due to the growing problem of honey adulteration, a rapid and accurate detection technique is required.

Due to its high specificity and sensitivity, PCR has been used as one of the DNA-based analysis techniques to detect honey adulteration with syrup (Sobrino-Gregorio, Vilanova, Prohens, & Escriche, 2019; Truong, Kim, & Yoon, 2022). However, PCR requires repeated thermal denaturation, and professional operation is not suitable for rapid on-site

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testing. Isothermal amplification technology has become an alternative method for point-of-care detection (POC) in recent years (Gloekler, Lim, Ida, & Frohme, 2021). Recombinant enzyme polymerase amplification (RPA) has become a suitable technique for species identification due to its simplicity, short reaction time, and low reaction temperature (Tian, Liu, Ye, Li, & Chao, 2017). RPA has been used to authenticate animal products, including meat (Cao et al., 2018; Ivanov, Popravko, Safenkova, Zvereva, Dzantiev, & Zherdev, 2021; James & Macdonald, 2015; Kissenkoetter, Boehlken-Fascher, Forrest, Piepenburg, Czerny, & Abd El Wahed, 2020) and seafood (Frigerio, Gorini, Palumbo, De Mattia, Labra, & Mezzasalma, 2023; Xie, Zhang, Wen, Zhao, Wang, & Chen, 2023). There are few reports of its use to detect honey adulteration. Compared with PCR detection technology, RPA is free from thermal cycling instruments, has a shortened amplification time, has simple operation, and has great potential for application as a tool for honey adulteration detection.

Nucleic acid extraction is another major bottleneck in the development of rapid field testing. As the demand for on-site testing increases, simple and rapid nucleic acid extraction methods are critical. Currently, the commonly used nucleic acid extraction methods are hexadecyl trimethyl ammonium bromide (CTAB) and commercial extraction kits. However, these techniques are time-consuming (1–2 h) and cumbersome (Garcia, Farnleitner, Mach, Krska, & Brunner, 2016; Sajib, Bhuiya, & Huque, 2017; Singh, Sodhi, Paliwal, Sharma, & Randhawa, 2021). Silica membrane is a good candidate material for nucleic acid extraction. DNA can be rapidly adsorbed and eluted using silica membranes at different salt concentrations (Wang, Yan, Wang, Zhao, Shi, & Ma, 2020; Zhao et al., 2021). In this study, we developed a rapid DNA extraction device consisting of a screw joint, an adsorption column, a waste tube, and a syringe. DNA was absorbed onto the silica membrane by applying pressure via a syringe to rapidly obtain DNA.

This study aimed to develop a REMD-RPA strategy for the rapid and accurate detection of corn syrup adulteration in honey. First, the procedure of REMD was described, followed by a feasibility evaluation. The established RPA was used to detect the *(ZssIIb)* gene and evaluate the specificity and sensitivity of the method. Finally, the feasibility and sensitivity of the REMD-RPA detection platform were evaluated. The objective was to develop a rapid and simple method for on-site detection of honey adulteration.

2. Materials and methods

2.1. Materials and sample preparation

Data regarding the sample preparation and materials used in this study are listed in Table S1. The primer and probe sequences are listed in Table S2.

2.2. DNA extraction

The rapid extraction method involves four devices: a screw joint, an adsorption column, a waste tube, and a syringe (Fig. 1). A mixture of 5400 μL of corn syrup and 7200 μL of lysis buffer (20 mM Tris-HCl, 2 mM EDTA, and 5 mM GuHCl) from the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was transferred to a filter cartridge. Then, the syringe plunger was pressed to filter the mixture through the filter membrane and into the adsorption column. The adsorption column was transferred to a new 2 mL test tube, and 500 μL of 75 % alcohol was passed through it to wash the silica membrane. Subsequently, the adsorption column was placed in a new 1.5 mL test tube, and 60 μL of TE buffer was added to elute the DNA on the silica membrane. DNA concentration was determined using a Nanodrop 2000C (Thermo Scientific Company, Delaware, DE, USA).

Concurrently, plant DNA extraction was performed as per the instructions in the DNeasy Plant Mini Kit manual (Qiagen, Hilden, Germany). The DNA concentration was then determined using a Nanodrop 2000C (Thermo Scientific Company, Delaware, DE, USA).

2.3. Recombinase polymerase amplification (RPA)

RPA was performed using a commercial kit from Amplification Futere (Weifang, Shandong, China). The procedure involved combining 29.4 μL of Buffer A, 2 μL of forward and reverse primers (10 nmol/L), 0.6 μL of probe (10 nmol/L), 2 μL of template DNA (51.4 ng/μL), one lyophilized enzyme pellet, and nuclease-free water. After thorough mixing, 2.5 μL of Buffer B was added. The RPA reaction strip was amplified at 39 ℃ for 20 min. An optimal combination of primers and probes was selected (Fig. S1 of primer screening). The real-time fluorescence signal of RPA with the Exo-probe was monitored using a Bio-Rad Detection System (Bio-Rad Company, Pleasanton, CA, United States). The visual green fluorescence result was obtained using glasses under a handheld lamp (LUYOR-3415RG), a process referred to as the VG-RPA.

2.4. Specificity and sensitivity of RPA detection

The specificity of RPA detection was assessed using genomic DNA from maize, rice, soybean, cotton, sugarcane, and Cole, with deionized water (ddH_2O) serving as the negative control.

To measure the sensitivity of RPA detection, the DNA concentration

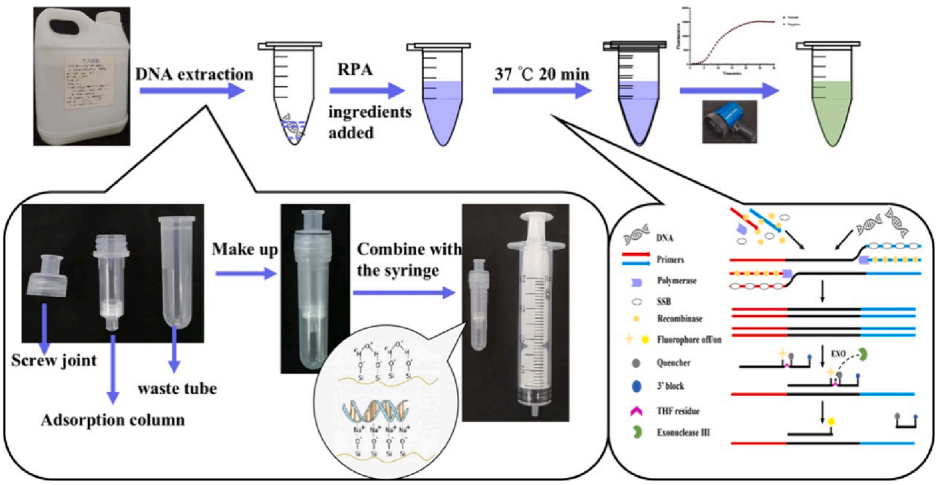


Fig. 1. Schematic diagram of the strategy for detecting syrup adulteration in honey based on RPA.

was determined using Nanodrop 2000C (Thermo Scientific Company, Delaware, DE, USA). The number of DNA copies was calculated using the following formula: DNA copy number (copies/μL) = (c(ng/μL) × 10^-9 × 6.02 × 10^23)/ (n × 660), where c and n represent DNA concentration and length, respectively. The extracted DNA was diluted to 8000 copies/μL and then subjected to a 5-fold serial dilution to 12 copies/μL, using ddH_2O as the diluent.

2.5. The RPA for corn syrup and honey mixtures detection

To verify the practicality of the established fluorescence RPA method for commercial products, both pure and artificially adulterated samples were selected for blind testing. The syrup was added to pure honey to obtain the following syrup-to-honey ratios (v/v): 80/20 (20 %), 90/10 (10 %), 95/5 (5 %), and 99/1 (1 %). These adulterations were designated as Adu1 to Adu4. The established RMED method was used to extract DNA. Finally, a fluorescence visualization analysis was conducted using the RPA amplification product. For the test of actual samples, each sample was tested in triplicate, and each test was repeated three times.

2.6. Statistical analysis

All experiments were repeated three times, and the mean and variance were calculated using Microsoft Excel (Microsoft Inc., Washington, DC, USA). The results were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA), with $p < 0.05$ considered statistically significant. Data were analyzed using the Origin software (Origin Lab, Northampton, MA, USA) and GraphPad Prism 8.

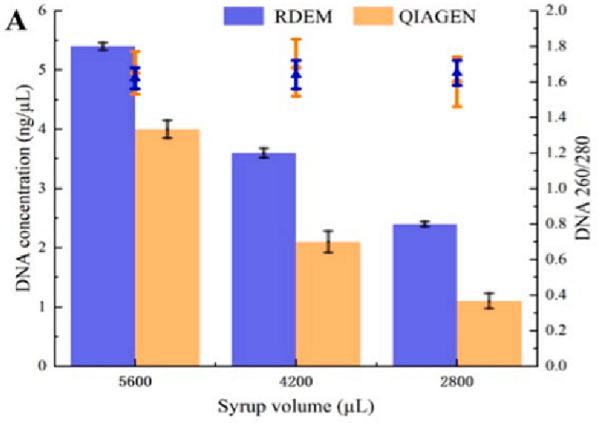
3. Results and discussion

3.1. Syrup nucleic acid DNA quality analysis

Extracting DNA from the complex matrix of honey and deeply processed syrup to obtain a usable quantity of DNA targets is challenging (Abdel-Latif & Osman, 2017; Aboul-Maaty & Oraby, 2019; Soares, Amaral, Oliveira, & Mafra, 2015). The conventional methods using CTAB or commercial kits are time-consuming, dependent on large-scale instruments, and more expensive (Guertler, Eicheldinger, Muschler, Goerlich, & Busch, 2014; Singh et al., 2021). Therefore, the first hurdle was to establish a rapid DNA extraction method, a prerequisite for amplification by RPA.

There was a significant difference in DNA concentration between the Rapid DNA Extraction Method (RDEM) and the QIAGEN kit extraction from syrup, with RDEM yielding higher results than the kit method, extracting 5.4 ng/μL DNA from 5600 μL of syrup (Fig. 2A). The difference in DNA purity between the two methods was negligible, with values below 1.8, due to the presence of unpurified sugar components in the syrup DNA (Shepherd & McLay, 2011). However, the two methods differed significantly in terms of extraction time and material cost. The QIAGEN method required 1 h, while the RDEM required 5 min (Fig. 2B). These results show that the DNA extracted by the rapid extraction method is effective.

To evaluate the efficacy of the Rapid DNA Extraction Method (RDEM) and the QIAGEN method for DNA extraction in subsequent experiments, PCR was used to amplify the plant endogenous gene 18S rRNA and the maize-specific gene *zssIIb,* extracted using these methods. The products were visualized by electrophoresis (Fig. 2C-D). Product amplification was visible at 151 and 181 bp. The brightness of the DNA amplification bands extracted using RDEM was almost the same as that



B

	RDEM	QIAGEN
Time of extraction	5 min	60 min
Cost of materials	10 $ (50 per)	400 $ (50 per)
Whether used instruments	Yes	No

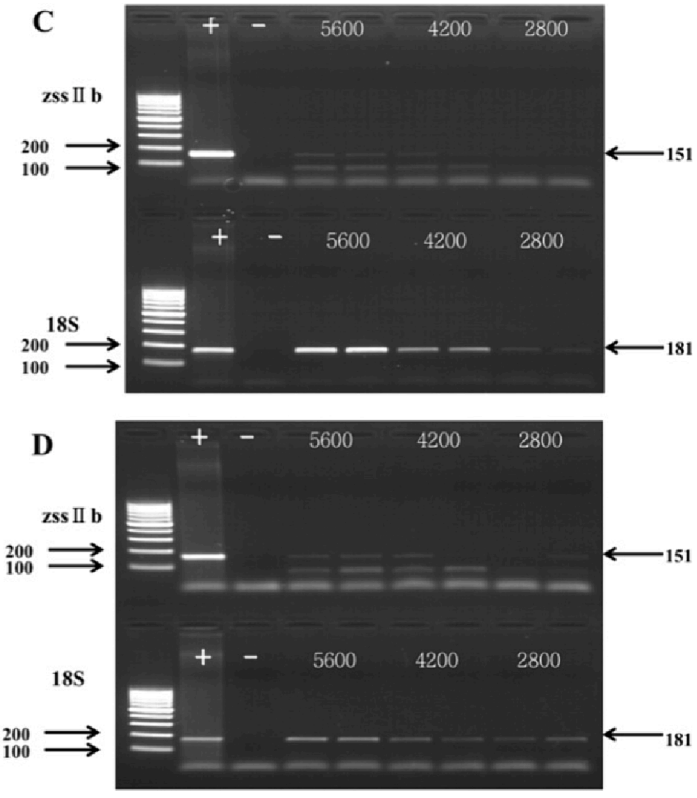


Fig. 2. Quality analysis of syrup DNA extracted by the QIAGEN and rapid extraction methods, as well as the results of ordinary PCR amplification and their differences, are compared. A. Analysis of DNA concentration and purity in syrup by two methods. B. Comparison of the differences between the two methods. C. Amplification results of DNA maize-specific genes *ZssIIb* and 18SrRNA extracted by rapid extraction method. D. Amplification results of DNA maize-specific genes *ZssIIb* and 18SrRNA extracted by QIAGEN method; M: DL 1000 DNA marker.

obtained using QIAGEN. We concluded that DNA extracted using RDEM could be used for subsequent PCR assays.

3.2. Sensitivity and specificity of RPA in species identification

To determine the sensitivity of RPA, its detection limit was measured. Extracted DNA was diluted to 8000 copies/μL, followed by 5-fold serial dilution to 12 copies/μL. RPA detected the maize target gene at a concentration of 12 copies/μL with a fluorescence value of approximately 100 RFU (Fig. 3A). These results were consistent with the visualization results (Fig. 3B). Then, maize genes were analyzed at different concentrations using PCR and qPCR. The intensity of the target bands and fluorescent signals diminished as the copy number decreased, and finally, 12 copies/μL were detected (Fig. 3C-D). These results are consistent with RPA results and indicate the potential nucleic acid quantification capability of the RPA method.

Highly specific primers only amplify the target bands, whereas non-target bands are not cross-amplified. To assess specificity, primers for other plant sources, including sugarcane, rice, soybean, cotton, sugar beets, and Cole, were designed. DNA from maize, sugarcane, rice, soybean, cotton, sugar beet, and Cole was detected via these primers using the RPA, visualization, PCR, and qPCR systems. PRA could detect maize target genes and emit fluorescent signals within 30 min without cross-reacting with the other plant-source genes (Fig. 4A), consistent with the visualization results (Fig. 4B). In contrast, qPCR and PCR require 90 min of thermocycling amplification to achieve the same results. After gel electrophoresis, only the maize-containing target genes showed clear bands (Fig. 4C). Consistent with previously reported results (Sobrino-Gregorio, Vargas, Chiralt, & Escriche, 2017), PCR is a reliable detection method. In the qPCR system, only those genes containing the maize target gene emitted a fluorescent signal at 40 cycles (Fig. 4D). RPA is free from thermal cycling, significantly reduces assay time, avoids aerosol contamination caused by opening the cap, and has good specificity. These results suggest that the established RPA method has high specificity and can be a suitable technique for honey adulteration detection.

3.3. RPA detects corn syrup

The feasibility of the RPA detection method was validated using corn as the positive control, corn syrup as the sample, and water as the negative control. All samples were generated fluorescence signals except for the negative control (Fig. 5A). The fluorescence of the positive control reached a value of 1500 RFU at 20 min.The fluorescence of corn syrup reached a value of 500 RFU at 20 min. Fig. 5C-D display the RPA amplification products of the positive control and corn syrup, respectively, where green fluorescence can be observed with the naked eye. Fig. 5B illustrates the qPCR results, which were consistent with the RPA and visualization results. These results indicate that the RPA method can be effectively used to detect corn syrup.

3.4. Simulation of actual sample adulteration

Since honey is commonly adulterated with corn syrup, we mixed honey with corn syrup at ratios of 80/20 (20 %), 90/10 (10 %), 95/5 (5 %), and 99/1 (1 %) to assess the REMD-RPA efficiency. We found that the REMD-RPA assay platform could detect 1 % corn syrup adulteration in honey (Fig. 6). As the percentage of adulteration decreased, the fluorescence value decreased accordingly (Fig. 6A), and 1 % adulteration could be detected with a fluorescence value of approximately 150 RFU, consistent with RPA visualization results (Fig. 6B). These results suggest that REMD-RPA is potentially applicable to the identification of honey adulteration.

The REMD-RPA method is independent of thermal cycles, simple, has a short reaction time, and has high specificity. Namin et al. (Namin, Yeasmin, Choi, & Jung, 2022) used PCR to identify the adulteration of Apis cerana and dorsal honey. This method needs to visualize the PCR products through electrophoresis, potentially leading to aerosol pollution during this process, making it easy to produce false positives. Wang et al. (Wang, Zhang, Wang, Zhang, Jiao, & Liu, 2020) established the LAMP method to detect adulteration in rape honey. However, the LAMP method requires multiple primers and has a complex procedure. The REMD-RPA detection method established in this experiment only

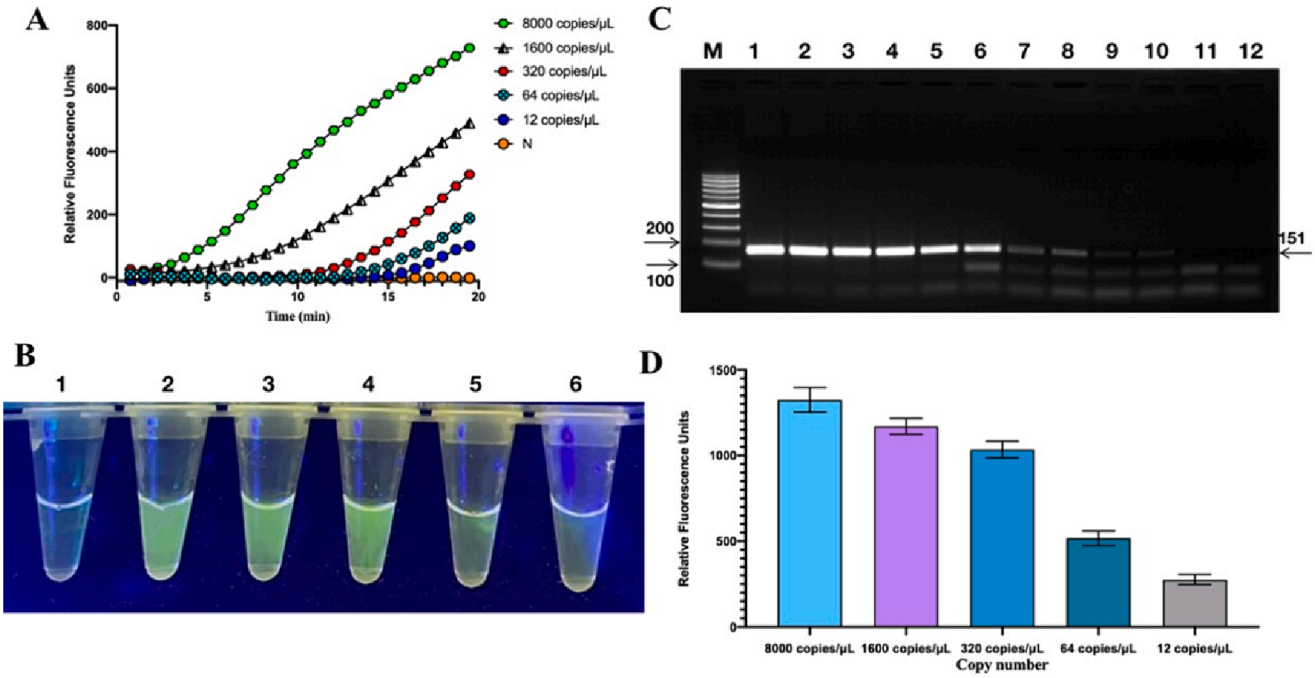


Fig. 3. Sensitivity of the RPA for maize detection. A. Real-time fluorescence of RPA at different copies (8000, 1600, 320, 64, and 12 copies/μL). B. Visual fluorescence after amplification (1: negative control, 2–6: 8000, 1600, 320, 64, and 12 copies/μL, respectively). C. Conventional PCR results of different concentrations of DNA (M: DL 1000 DNA marker; 1–2: 8000 copies/μL; 3–4: 1600 copies/μL; 5–6: 320 copies/μL; 7–8: 64 copies/μL; 9–10: 12 copies/μL; 11–12: water). D. Sensitivity of qPCR for various concentrations of the maize genome at 40 cycles.

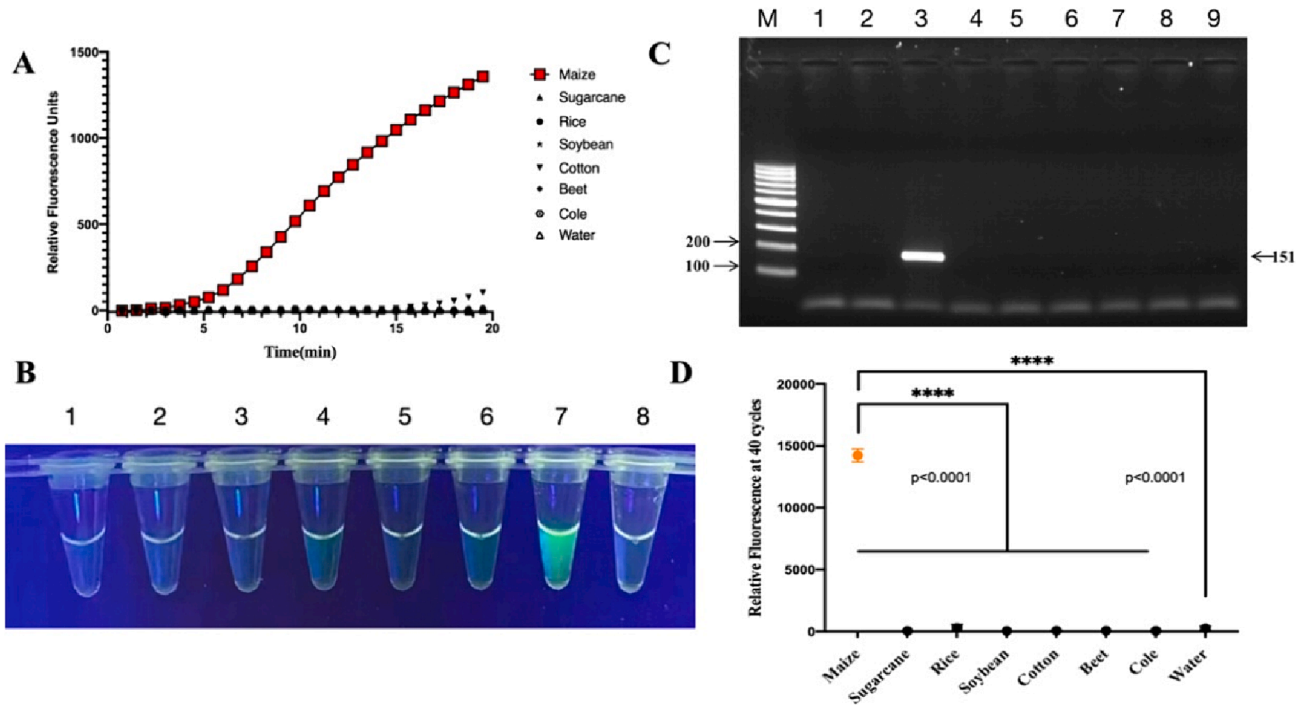


Fig. 4. Specificity of the RPA for maize detection. A. Real-time fluorescent RPA detection. B. Visual fluorescence detection (1–8: water, Cole, cotton, sugarcane, rice, soybean, maize, and beet, respectively). C. Conventional PCR (M: DL 1000 DNA marker; 1–2: negative control; 3–9: maize, Cole, cotton, rice, beet, soybean, and sugarcane, respectively). D. Specificity of qPCR for the maize genome at 40 cycles.

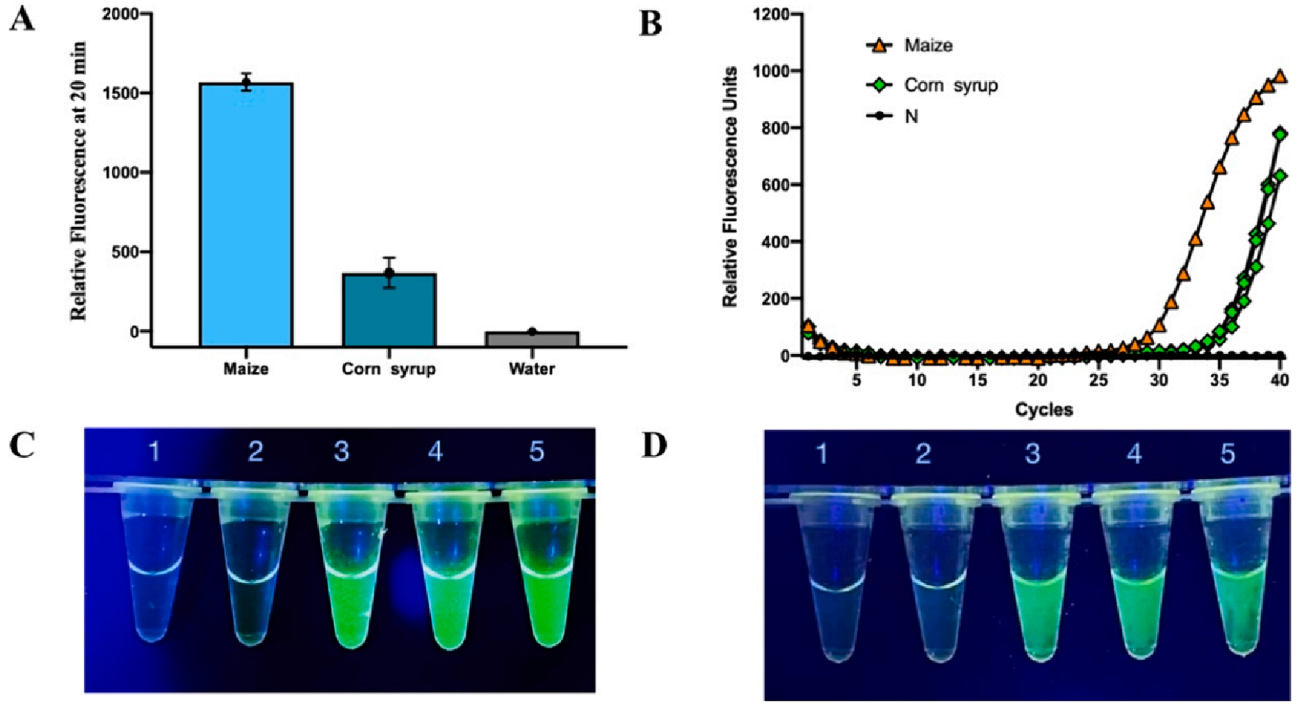


Fig. 5. RPA detects corn syrup adulteration. A. The RPA for corn syrup detection at 20 min. B. qPCR. C. Visual fluorescence after amplification of the maize genome (Tube 1–2: negative control; 3–5: maize). D. Visual fluorescence after amplification of corn syrup (Tube 1–2: negative control; 3–5: corn syrup).

required the design of a pair of primers without opening the cover to avoid false positives.

3.5. Practical detection in products

The well-known standard method for authenticating honey is melissopalynology (i.e., analysis of pollen grains contained in honey deposits using optical microscopy) (Louveaux, Maurizio, & Vorwohl, 1978). To verify whether the samples contained pollen of the tested plants either naturally or intentionally added by air transmission, we used traditional melissopalynology and PCR methods to verify the purity of the samples. As presented in Fig. S2, the detected canola honey was

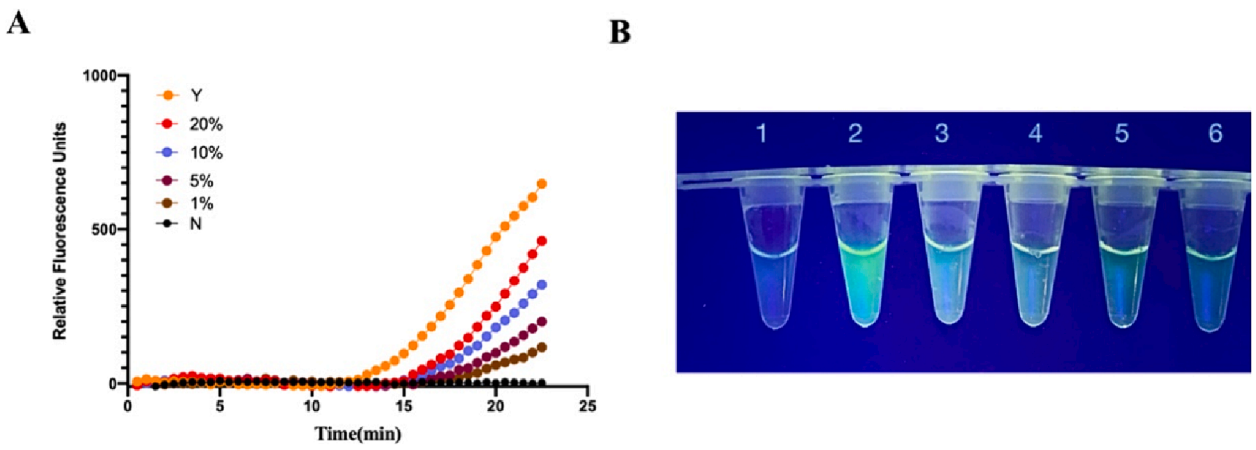


Fig. 6. Simulation of actual sample adulteration. A. Practicality of the RPA method for detecting honey adulteration. Different percentages (20%, 10%, 5%, and 1%) of corn syrup were added to honey to simulate real-life adulteration of honey products. B. Visual fluorescence after amplification (the adulterated honey concentrations selected for tubes 1–6 were negative, positive control (maize), 20%, 10%, 5%, and 1%).

observed by melissopalynology, and the results indicated that it contained only canola pollen but not maize pollen, consistent with PCR results. These results indicate that pure canola honey was used as the sample.

Twenty-two honey samples purchased from the market were used to validate the potential application of the REMD-RPA assay in honey syrup adulteration. The data in Table 1 demonstrate that 7 out of 22 samples contained corn syrup adulteration, with a positive rate of 31.82 %, consistent with visualization results (Table S3). The 100 % positive detection rate of REMD-RPA assay was consistent with the detection rate of PCR, indicating the good performance of this method. These results suggest that the REMD-RPA assay can be used to detect adulteration in honey.

4. Conclusion

We successfully developed a rapid nucleic acid extraction method from molasses that, in combination with RPA technology, accurately detects corn residue DNA in honey. A novel rapid DNA extraction method was used that takes about 5 min to extract DNA from corn syrup. The entire test takes less than 30 min. Additionally, RPA showed high accuracy (LOD of 12 copies/μL) and specificity (no non-specific amplification with other plant genomes) in detecting endogenous genes of maize. REMD-RPA can detect 1 % adulteration of corn syrup in honey. Moreover, the method showed good applicability in detecting real samples. Consequently, REMD-RPA is a rapid, accurate, and specific detection method that provides technical support for rapid on-site detection of honey adulteration and market supervision.

Table 1
Direct detection of corn syrup adulteration in commercially available honey using RPA, fluorescence visualization, and PCR. Data are expressed as number and percentage (%) of specimens.

REMD-based RPA	VG- RPA		PCR		Total
	Positive n = 7	Negative n = 15	Positive n = 7	Negative n = 15	
Positive	7 (100 %)	0 (0 %)	7 (100 %)	0 (0 %)	7 (31.82 %)
Negative	0 (0 %)	15 (100 %)	0 (0 %)	15 (100 %)	15 (68.18 %)
Total	7 (31.82 %)	15 (68.18 %)	7 (31.82 %)	15 (68.18 %)	22 (100 %)

CRediT authorship contribution statement

Huixing Ye: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Wenqiang Chen:** Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Tao Huang:** Supervision, Resources, Funding acquisition, Conceptualization. **Junfeng Xu:** Supervision, Resources, Funding acquisition, Conceptualization. **Xiaofu Wang:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2024.100206.

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