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Comparative evaluation of various DNA extraction methods and analysis of DNA degradation levels in commercially marketed Chestnut rose juices and beverages

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Abstract

Background Food safety is a significant global study subject that is strongly intertwined with human life and well-being. The utilization of DNA-based methods for species identification is a valuable instrument in the field of food inspection and regulation. It is particularly significant for traceability purposes, as it enables the monitoring of a specific item at every level of the food chain regulation. However, obtaining amplifiable genomic DNA in this process is a significant obstacle in gene studies. To date, there is a lack of literature on DNA extraction from processed juice or beverages, and no data exist on simultaneous comparisons of various extraction processes. This study aimed to optimize and compare four DNA extraction methods for Chestnut rose juices and beverages. Furthermore, we also conducted a comparison and analysis of the extent of DNA degradation in Chestnut rose juice or beverage by utilizing the amplicon size.

Methods The quantity and quality of the extracted DNA were assessed using NanoDrop One spectrophotometer, gel electrophoresis, and real-time polymerase chain reaction (real-time PCR or qPCR) assays. An assessment was conducted on the processing time, labor intensity, and cost associated with each approach. The degree of DNA degradation in Chestnut rose juice or beverage was also assessed using TaqMan real-time PCR methods.

Results The non-commercial modified CTAB-based approach yielded a high DNA concentration. However, spectrophotometric results and real-time PCR analysis showed poor DNA quality. The combination approach showed the greatest performance among the extraction methods, while being comparatively time-consuming and costly in contrast to the other methods. Additionally, the analytical findings of DNA degradation suggested that the integrity of sample DNA could be influenced by the intricacy of processing methods used by various manufacturers.

Conclusions To achieve precise DNA quantification, selecting suitable extraction strategies for the given matrix is necessary. The combination approach was identified as the most effective DNA extraction technique and is suggested

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for extracting DNA from Chestnut rose juices and beverages. This comparative assessment can be particularly valuable for extracting and identifying processed Juices and Beverages in a diverse range of food compositions.

Keywords Food safety, DNA extraction procedures, DNA degradation extent, Spectrophotometric NanoDrop approach, Gel electrophoresis technique, Real-time PCR technology, Chestnut rose juices and beverages

Introduction

The focus on guaranteeing food quality and safety management has increased due to consumer concerns, corporate strategies, and government policy initiatives. In 1974, the United Nations Food and Agriculture Organization (FAO) raised global awareness for the first time during the World Food Summit by introducing the concept of "food security." Subsequently, nations worldwide placed significant emphasis on the advancement of this domain. In China, the 1995 food safety legislation granted the Ministry of Health the authority to supervise food safety by regulating food labeling, quality, safety, and packaging. Ensuring food traceability from the farm to consumers, employing tests to identify and avoid food safety risks, and maintaining the integrity and quality of new food products are crucial components of a reliable agricultural supply chain management system [1]. Chestnut rose (*Rosa roxburghii* Tratt), commonly called Cili in China, is a fruit-bearing plant cultivated in the mountainous regions of southwest China. It thrives at elevations ranging from 500 to 2500 m in provinces such as Guizhou, Sichuan, and Yunnan [2, 3]. Guizhou, which is the primary region for cultivating Chestnut roses in China, has experienced significant growth in this industry. By the end of 2022, the cultivation area has expanded to over 210 thousand hectares. Additionally, the yearly production of Chestnut rose fruit in Guizhou provinces has reached nearly 300,000 tons [4]. Scientists have discovered numerous nutritional and medicinal elements in the fruit, including phenolics, polysaccharides, triterpenoids, superoxide dismutase, proteins, vitamins, ascorbic acid, amino acids, fatty acids, and other organic acids. These components have been proven to possess antioxidant, antiatherogenic, hypoglycemic, anti-aging, and antitumor properties [5, 6]. In recent years, Chestnut rose fruit has been extensively used and harnessed in the food business to produce various types of Chestnut rose juices or beverages, owing to its potential nutritional and functional characteristics [6]. However, processed Chestnut rose juice or beverages are especially susceptible to fraudulent activities due to the market's large size, the great profitability of these items, and the challenges associated with identifying species in processed Chestnut rose products [7]. These fraudulent practices result in financial harm to consumers, and certain types of fraud, such as mislabeling or species substitution, can pose significant health risks (toxicity and allergenicity). Therefore, ensuring the traceability of raw materials derived from the Chestnut rose plant and preventing the adulteration of Chestnut rose in juices or beverages are crucial for safeguarding public health and promoting fair trade.

Currently, several techniques have been utilized to trace and identify the adulteration of processed products. The practice of morphological identification involves sensory assessment of shape, color, odor, and texture [8], as well as microscopic examination of tissue structure and arrangement of raw materials [9]. While these methods may be uncomplicated and inexpensive, their accuracy heavily relies on expertise and proficiency, making it challenging to distinguish between closely related alternatives. Additionally, several protein detection technologies are being utilized, including electrophoresis, chromatography, and immunology [10]. Furthermore, a range of spectrometric instruments, including high-performance liquid chromatography (HPLC) [11], liquid chromatography-tandem mass spectrometry (LC-MS-MS) [12], nuclear magnetic resonance (NMR) [13], Fourier transform infrared spectrometer (FTIR Spectrometer) [14], and mass spectrometry (MS) [15], are utilized to examine metabolites in the processed products. While these methods were effective in analyzing the components in fruit juice, they were susceptible to various factors such as cultivar, growing region, harvest maturity [16], cultivation practices, storage atmosphere [17], climate, storage conditions [18], processing [19], and shipping [20], which could impact the accuracy of species identification [21]. In contrast, the polymerase chain reaction (PCR) techniques, which are based on DNA, offer a viable alternative because of their exceptional sensitivity and specificity. These techniques enable the identification of minuscule quantities of DNA in raw materials and processed foods. DNA amplification methods are increasingly becoming recognized as valuable approaches in the field of food inspection and regulation. These methods are not only capable of detecting different species in fruit juice [22] but also identifying instances of food adulteration [23]. Their potential is vast and inspiring, promising a new era in food inspection and regulation and instilling hope for a safer and more authentic food supply.

However, the effectiveness of DNA amplification methods relies on the efficacy of DNA extraction protocols, which should yield a substantial amount of high-quality DNA. In addition to being efficient, appropriate extraction methods should also be user-friendly, economical, and time-saving. Ideally, these methods can be applied universally to minimize the need for several extraction

procedures and calibrations in Real-time PCR, DNA quantification following gel electrophoresis, or spectrophotometric quantification. Undoubtedly, the utilization of PCR relies on the isolation of DNA from diverse dietary substances, which is frequently the most crucial stage. It is important to acknowledge that thequality of DNA extracted from food samples is influenced by various factors. These include the presence of PCR inhibitors such as polysaccharides, polyphenols, and proteins in the food matrices [24], as well as DNA polymerase inhibitors like tannins, alkaloids, and polyphenols [25]. The quality is also influenced by the extent of DNA damage, such as depurination, and the average length of the nucleic acid fragments obtained [26]. These aspects rely on the sample itself, the procedures used during food preparation, and the physical and chemical parameters of the extraction method employed. To be more precise, the presence of complex matrices in Chestnut rose juice or beverages, as mentioned beforehand, can hinder the amplification of isolated DNA. In addition, Chestnut rose juice or beverages have often undergone several processing stages, including mechanical, thermal, chemical, or enzymatic treatment, which have impacted the integrity of DNA. Once again, DNA exhibits a high susceptibility to acid due to the process of hydrolytic destruction. A comprehensive analysis using ultra-fast liquid chromatography/quadrupole time-of-flight mass spectrometry (UFLC/Q-TOF-MS) has identified a total of 13 organic acids in Chestnut rose fruit, including ascorbic acid, malic acid, lactic acid, gallic acid, citric acid, p-coumaric acid, protocatechuic acid, syringic acid, 9,12,15-octadecatrienoic acid, p-hydroxybenzoic acid, caffeic acid, and 9,12-octadecadienoic acid [27]. The acidity present in fruits enhances the pace of acid-catalyzed reactions during heat treatments [28]. Moreover, the Chestnut rose juice or beverages underwent a packaging and canning process that involved various thermal procedures and pressure application before being introduced to the global market. The filling medium is also recognized as the main cause of DNA degradation in canned food products [29]. Therefore, there is an urgent need for improved and efficient DNA extraction methodologies and more accurate methods to assess the quantity and quality of the extracted DNA and ensure the safety and authenticity of our food supply.

Numerous methods are available for extracting DNA [30–32], but only a few can be utilized to isolate DNA from processed food products [33]. In addition, there has been limited comparison of various existing DNA extraction procedures in a comprehensive manner, particularly in Chestnut rose juices or beverages. In this current undertaking, the purity, quality, and quantity of DNA extracted from Chestnut rose juice or beverages were compared using two regularly used commercial methods, one non-commercial method, and one combined DNA extraction method. In particular, the quantity of isolated DNA was assessed using a nanodrop spectrophotometric technique. To assess the amplification level of the isolated DNA, we conducted conventional PCR and real-time PCR analyses using specific primers for the internal transcribed spacer 2 (ITS2), a nuclear ribosomal gene region specific to the Chestnut rose. In addition, we also critically assessed the handling technique, time consumption, expenses per preparation, and the convenience of establishing the extraction procedures in our laboratory. Furthermore, the degree of DNA degradation in Chestnut rose juice or beverage was measured using PCR amplification with primers that yielded amplicons of various sizes. This study may aid the identification of species in processed fruit juices and beverages to safeguard manufacturers from unfair competition and consumers from fraud and adulteration.

Table 1 Characteristics of the sample materials used in the study

Sample group	Description(content)	Sample ID	Manufacturer
Chestnut rose juice	100% Chestnut rose juice	C1	Guizhou Guiding Min Zi food Co., LTD
Chestnut rose juice	100% Chestnut rose juice	C2	Guizhou Chuhao agricultural science and technology development Co., LTD
Chestnut rose juice	100% Chestnut rose juice	C3	Guizhou Hengmaoyuan Biotechnology Co., LTD
Chestnut rose juice	100% Chestnut rose juice	C4	Guizhou Sanwongkwo Healthy Industry Co., LTD
Chestnut rose beverage	30% Chestnut rose juice (70% consists of pure water and food additives.)	C5	Guizhou Hongcaijunong Investment Co., LTD

Materials and methods

Samples

This study collected individual samples from five Chestnut rose juice or beverage producers located throughout Guizhou province. Table 1 lists the names, brief descriptions, brands, and manufacturers concerning each sample. We marked these five commercially marketed Chestnut rose juice or beverages as "C1", "C2", "C3", "C4" and "C5", respectively. These samples were kept at 4 °C and were used to evaluate the DNA extraction methods described next. The Chestnut rose fruits used as positive controls were collected from Longli County, Qiannan Prefecture, Guizhou province, and were verified by professionals from Guizhou Qiannan Inspection and Testing Institute.

DNA extraction methods

Total DNA was extracted from these samples using four distinct DNA extraction methods. Two commercial methods were utilized in the analysis: Plant Genomic DNA Kit (abbreviated as PG) and Magnetic Plant Genomic DNA Kit (abbreviated as MPG) (TianGen™, Beijing, China). One previously published noncommercial method was also evaluated: the cetyltrimethylammonium bromide (CTAB)-based method ("CTAB") [34], which has been modified in our laboratory (abbreviated as MC). In addition, one combined method was used in the evaluation: isopropyl alcohol precipitation combined with Processed Food DNA Extraction Kit (abbreviated as IPF) (TianGen™, Beijing, China). The four DNA extraction methods that were used for this investigation are listed in Table 2 along with their key features. All DNA extracts were suspended in a final volume of 50 µL elution buffer, and all extractions were carried out by a single individual in order to fairly assess and compare the performance of these extraction techniques.

Commercial method

For DNA extraction with PG and MPG methods, the samples (2 mL of juice or 5 mL of the beverage) were centrifuged at 13,400×g (equivalent to approximately 12000 rpm) for 5 min, and the supernatant was discarded. The deposit was managed according to the manufacturer's usual procedure.

Noncommercial method

The MC approach has three primary stages: sample extraction, DNA purification, and DNA precipitation. The genomic DNA extraction process involved pretreating samples (2 mL of juice or 5 mL of the beverage) using the previously described methods (Reference the "Commercial method" section). The supernatant was transferred, and the sediment was combined with 700 µL of 2×CTAB extraction buffer (containing 20 g/L CTAB, 1.4 mol/L NaCl, 0.02 mol/L ethylenediaminetetraacetic acid (EDTA) at pH 8.0, and 0.1 mol/L Tris-HCL at pH 8.0). The mixture was vortexed for 10 to 20 s to disperse any clumps, followed by incubation at 65 °C for one hour. The tube was then repeatedly inverted multiple times to ensure thorough mixing. Subsequently, an equivalent amount of a water-saturated mixture containing phenol, chloroform, and isoamyl alcohol in a ratio of 25:24:1 was introduced. The resulting solution was well mixed using vortexing and then subjected to centrifugation with a force of 13,400×g for a duration of 5 min. After that, the organic phase was disposed away, while the aqueous phase underwent a further extraction with 700 µL of trichloromethane using identical procedures. The supernatant was collected and combined with an equal volume of isopropyl alcohol. The mixture was thoroughly mixed and then stored in a refrigerator at a temperature of -20 ℃ for a duration of 1 h. Following this, the mixture was subjected to centrifugation at a speed of 13,400×g for a duration of 5 min. The liquid portion above the sediment was removed, and the sediment was rinsed with 700 µL of 75% ethanol. The mixture was then stirred for 5 s using a vortex mixer and centrifuged at a speed of 13,400×g for a duration of 2 min. The ethanol solution was carefully discarded, and this washing process was repeated. Finally, the cap was then opened, and the remaining ethanol solution was allowed to completely evaporate. The sediment was subsequently dissolved in a 50µL solution of TE (Trishydrochloride buffer, pH 8.0, containing 1.0 mM EDTA) and stored at a temperature of -20 ℃ for future use.

Combined method

The last one, the MC approach, has been adapted in our laboratory in the following manner: A volume of 2 mL of juice or 5 mL of the beverage was subjected to centrifugation at a speed of 10,000×g (equivalent to approximately 10000 rpm) for a duration of 15 min. The resulting supernatant was discarded, and subsequently, 1 mL of a Tris.Cl buffer with a concentration of 0.1 M and a pH of

Table 2 Key characteristics of the four selected DNA extraction procedures used in this study

	Extraction protocol	Kit name abbreviation	Cell lysis	Extraction buffer	Elution buffer	DNA purification	Special advantages
Noncommercial method	Modified CTAB method	MC	CTAB	20 g/L CTAB、1.4 mol/L NaCl、0.02 mol/L EDTA(pH8.0)、0.1 mol/L Tris-HCL(p H8.0)	50 µL TE (pH 8.0)	Isopropanol	Economical; widely used
Commercial methods	Plant Genomic DNA Kit	PG	CTAB	700 µL 65 °C preheated GP1 and 0.1% β-mercaptoethanol	50–200 ul TE	Spin Columns CB3	Fast; simple; convenient; widely used
	Magnetic Plant Genomic DNA Kit	MPG	SDS, RNase A	400 ul buffer GPM and 5 ul RNase A (10 mg/ml)	50–100 ul TB	Isopropanol/ MagAttract Suspension G	Rapid and convenient
Combined method	isopropyl alcohol precipitation combined with Processed Food DNA Extraction Kit	IPF	Proteinase K, SDS	500 ul buffer GMO1 and 20 ul Proteinase K (20 mg/ml)	20–50 ul TE	Isopropanol	Safe; convenient; excellent scalability and flexibility

8.0 was added to these samples. This washing step was repeated three times, with each cycle involving vortexing to ensure thorough mixing, followed by centrifugation at 10,000×g for 15 min. After discarding the supernatant, the resulting precipitate was deemed suitable for further use. A total of 500 µL of Buffer GMO1 (DP326; TianGen™, Beijing, China) and 20 µL of Proteinase K (20 mg/ml) were introduced to the previously treated samples. The mixture was vigorously vortexed for a duration of 1 min, followed by an incubation period at 56 °C for 1 h. Throughout this incubation, the samples were periodically shaken every 15 min. Subsequently, 200 µL of Buffer GMO2 (DP326; TianGen™, Beijing, China) was introduced and subjected to vortexing for a duration of 1 min. The resulting samples were then maintained at ambient temperature (15–30 °C) for a period of 10 min. Following centrifugation at a speed of 13,400×g for a duration of 5 min, the supernatant was carefully transferred to a fresh centrifuge tube. Then, a volume equivalent to 0.7 of the supernatant was supplemented with isopropanol and thoroughly mixed using a vortex. In this experimental procedure, the mixing pre-cooling step was conducted at a temperature of -20 ℃ for a duration of 1 h to induce the precipitation of DNA. The mixture underwent centrifugation at a speed of 13,400×g for a duration of 3 min, after which the supernatant was removed. Then, the manufacturer's protocol was followed.

Spectrophotometer measurements

DNA yield and quality were measured after extraction using different methods with a UV-Vis spectrometer (NanoDrop™ One, Thermo Scientific, Thermo Fisher, Waltham, MA, USA). The assessment of protein contamination was conducted by determining the ratio of absorbance at 260 nm to that at 280 nm. Similarly, guanidine contamination was assessed by calculating the absorbance ratio at 260 nm to that at 230 nm. Each extract is averaged across numerous measurements to minimize mistakes. Each experimental trial was conducted with a sample volume of 1 µL, with the elution buffers from commercially available kits acting as the control, while sterile double-distilled water (dd H2O) functioned as the blank.

Table 3 Primers and probe specific for Chestnut rose juice and beverage

Target	Primer and probe names	Sequence (5′–3′)	Annealing temp (°C)	Amplicon length (bp)
ITS2	F94	CACGACAATCGGTGGTTGTCA	57.57	94
	F186	TCGGGAGTTGGATGGGACG	59.48	186
	F301	CTTGGTGTGAATTGCAGAA TCC	55.81	301
	F449	CCAAGGAACTTGAATGAAA GAGC	55.99	449
	F500	CTTGCGCTTGATCGACCCTC	59.50	500
	R	GCATCGACGGATCGACAC GTAT	59.54	
	P	FAM-AGAAAGCACTCGATCAA CACGAGCG- BHQ	62.20	

PCR amplification

The ITS2 region of nuclear ribosomal DNA (nrDNA) is considered a DNA marker in plant phylogenetic and DNA barcoding studies due to its valuable characteristics. These include the presence of conserved regions that allow for the design of universal primers, the ease of amplification, the ability to verify sequence errors through secondary structure alignments, and enough variability to differentiate closely related species [35, 36]. It is highly suggested as a gold standard barcode for identifying plants and fungi [37]. The researchers created and employed primers (forward primer F94: 5'- C ACGACAATCGGTGGTTGTCA-3'; reverse primer R: 5'-GCATCGACGGATCGACACGTAT-3') targeting the DNA sequences (Genbank Accession No. FJ358704, MH711604) of the ITS2 gene [38] in the Chestnut rose. This approach amplified a PCR product of 94 base pairs in length. Subsequently, the primers were employed to amplify the DNA isolated from all matrices using all the investigated procedures. To assess the rate of DNA degradation in commercially available Chestnut rose juice and beverages, we employed a universal reverse primer positioned in the downstream region of the ITS2 gene. Additionally, we utilized four different forward primers located in various upstream regions to generate DNA fragments of specific lengths, including 186 bp, 301 bp, 449 bp, and 500 bp. The primers were designed using SnapGene (version 7.0.2) software and verified them using the Primer-blast online server to identify potential mismatches in the genome. The oligonucleotides were produced and refined by Beijing Tsingke Biotech Co., Ltd. Table 3 provides the primer sequences, annealing conditions, and amplicon size.

For PCR analysis, reaction mixture was performed in a 25 µL reaction volume containing 12.5 µL of 2 × Taq PCR MasterMix (Solarbio Biotech, Beijing, China), 0.5 µL of each primer (10µmol/L), 2.0 µL template DNA, and 9.5 µL ddH_2O. PCR was conducted using the Veriti 96-Well Thermal Cycler. The process of DNA amplification was conducted utilizing the subsequent conditions: an initial denaturation step at a temperature of 94 °C for a duration of 2 min, followed by 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. The amplification process concluded with a final extension step at 72 °C for 2 min (Table 4). A PCR control was conducted using distilled water in place of DNA during the PCR reaction in order to assess and

Table 4 PCR program for amplification of all used genes

	Step		Temperature	Time
PCR	Initial denaturation		94 °C	2 min
	35cycles	Denaturation	94 °C	30 s
		Annealing	58 °C	30 s
		Extension	72 °C	30 s
	Final extension		72 °C	3 min
	Cooling		4 °C	∞
qPCR	UDG enzyme actiton		37 °C	120 s
	Prenaturation		95 °C	1 min
	40cycles	Denaturation	95 °C	10 s
		Annealing/Extension	60 °C	20 s

monitor any contamination in the PCR reagents. Following the process of PCR amplification, a volume of 5 µL was extracted from each PCR product and combined with an equal volume of DL500 DNA marker (manufactured by TaKaRa, Kyoto, Japan). This resulting mixture was subsequently loaded onto a 3.0% agarose gel. The gels were electrophoresed in a 1× TAE buffer supplemented with GoldView I nuclear staining dyes (Solarbio, Beijing, China) for a duration of 25 min at a voltage of 130 V.

Real-time PCR (qPCR)

This study also designed a real-time PCR assay to target the ITS2 gene region. This assay aimed to evaluate the presence of amplifiable DNA in extracts derived from Chestnut rose samples (C1, C2, C3, C4, C5). The sequences of primers mentioned above and probes employed are documented in Table 3. 5′-Ends of the probes were labeled with the reporter dye FAM (6-carboxyfluorescein), and 3′-ends were coupled to the black hole quencher I (BHQ I). The real-time PCR (qPCR) amplification was conducted using 96-well plates and analyzed using a LightCycler® 96 real-time PCR system (Light Cycler 96, Roche Diagnostics, Mannheim, Germany) manufactured by Roche in Germany. The real-time PCR reaction mixture was conducted with a final volume of 20 µL. This mixture consisted of 10 µL of 2 × TOROIVD® 5G qPCR Premix with UNG, 2 µl of the DNA sample, 0.5µL of each primer (10µM), 0.2µL of the specified probe (10µM), and ddH2O was added to reach the total volume. Standard cycling conditions included a UDG (uracil–DNA glycosylase) enzyme action at 37 °C (120s) and a Prenaturation step at 95 °C (1 min), followed by 40 cycles of denaturation at 95 °C (10 s), annealing/extension at 60 °C (20 s). The experimental protocol involved conducting a series of cycling steps under standardized conditions. These conditions encompassed the activation of a UDG enzyme at a temperature of 37 °C for a duration of 120 s, as well as an initial denaturationphase at 95 °C for a period of 1 min. Subsequently, a total of 40 cycles were performed, each consisting of denaturation at 95 °C for 10 s, followed by annealing and extension at 60 °C for 20 s. Table 4 presents the real-time PCR (qPCR) protocol employed for amplifying all the genes utilized in the study. In addition, positive controls were utilized by extracting DNA from the fruit of the Chestnut rose plant. Distilled water was utilized as a negative control without the presence of a DNA template. The LightCycler@ 96 Application Software employs specified dye-specific fluorescence threshold values to determine the Cq value of a sample. The threshold cycle (Cq) values acquired during the procedure evaluated the amplifiable DNA quantity obtained. In this context, smaller values are considered preferable.

Economic evaluation

Each DNA extraction approach was also evaluated based on the time and cost involved, as well as the complexity of its operation. The necessary duration was determined by adding the times required for each protocol stage, such as centrifugations and incubations. The allocation of funds for the comprehensive analysis was determined in light of the current expenses for consumables and reagents in China (see Table 5 for the column "Reagents and consumables"). Concurrently, these computations were performed on four extracted samples. In the course of the comparison, the material prices for each method were also computed.

Statistical analysis

The experimental data for the various DNA extraction procedures were evaluated using a general linear model

Table 5 Estimation of the labor intensity, time and material budgets required for each extraction procedure

Extraction protocol	Time (min)	Labor requirements (¥)	Reagents and consumables* (¥)	Estimated price per kit** (¥)	Total cost per sample (¥)
MC	163	"++"	3.53	/	3.53
PG	56	"+"	3.24	¥420 for 50 preps,¥1500 for 200 preps	10.74 ~ 11.64
MPG	56	"+"	1.79	¥760 for 50 preps,¥2880 for 200 preps	16.19 ~ 16.99
IPF	217	"+"	1.82	¥1200 for 100 preps	13.82

* Reagents and consumables include chemicals, gloves, tubes, tips, etc. **The data is provided by the manufacturer. (+) easy; (++) difficult

procedure, following the Psifidi et al. [39] model with certain changes. Each criterion for DNA evaluation (DNA yield, two spectrophotometer measurements, real-time PCR findings) was evaluated individually. We conducted a more stringent statistical analysis, wherein, if the data failed to conform to a normal distribution, we attempted a logarithmic modification of the data to align it more closely with a normal distribution.

$$\mathbf{Y_{ij}} = \mathbf{P_i} + \mathbf{e_{ij}} + \mu + \mathbf{q_j} \qquad (\mathbf{Model})$$

where $\mathbf{Y_{ij}}$= DNA score by assessment criterion for the $\mathbf{j^{th}}$ sample of the $\mathbf{i^{th}}$ extraction procedure, $\mathbf{P_i}$= effect of the $\mathbf{i^{th}}$ DNA extraction procedure ($\mathbf{i} = 1,\ldots,4$), $\mathbf{e_{ij}}$ = random residual, $\boldsymbol{\mu}$ = overall mean, $\mathbf{q_j}$= effect of the $\mathbf{j^{th}}$ sample.

To test for the homogeneity of variances, we run an F-test of equality of variances. Post-hoc analyses were conducted using least squares mean tests and a Bonferroni adjustment for multiple testing to compare various DNA extraction procedures for each evaluation criterion. A significance level of $p < 0.05$ was deemed as statistically significant. All analyses were conducted using IBM SPSS Statistics 20 (IBM, New York, NY, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Method validation

Each sample underwent three extractions to assess the repeatability and reliability of each extraction procedure. Each sample is assuredly drawn from a singular sample pool, ensuring equitable representation of all samples. Additionally, the extraction procedures were executed with precautionary measures to minimize sample contamination. The values displayed are the mean of all replicates ± standard deviation (SD).

Table 6 Evaluation and comparison of genomic DNA yield, purity and real-time PCR parameters (Cqvalue) obtained from different sample groups with the different extraction methods (MC: modified CTAB method, PG: plant genomic DNA kit, MPG: magnetic plant genomic DNA kit, IPF: isopropyl alcohol precipitation combined with processed food DNA extraction kit); values are given as mean value ± standard deviation of triplicates

Extraction method	DNA yield[1] (ng/μL)	A260/A280[2]	A260/A230[1,2]	Cq value[3]
MC	122.01 ± 29.3[a]	1.57 ± 0.09[a]	1.99 ± 0.09[a]	25.83 ± 1.36[b]
PG	14.26 ± 3.43[b]	1.72 ± 0.19[a]	1.43 ± 0.12[b]	26.24 ± 1.40[b]
MPG	11.23 ± 1.48[b]	0.99 ± 0.07[b]	0.54 ± 0.05[c]	27.84 ± 1.25[a]
IPF	187.62 ± 34.6[a]	1.74 ± 0.08[a]	2.14 ± 0.11[a]	22.7 ± 1.60[c]

[1] The ideal values are higher values

[2] A260/A280 and A260/A230 are ratios of absorbances measured at 260 nm and 280 nm, and at 260 nm and 230 nm, respectively

[3] The ideal values are lower values

[a, b,c, d] Comparison of each column's values

Suggested changes

Values (means ± SD, $n = 3$) with different letters are significantly different according to the Bonferroni test, $p < 0.05$

Results

The yield and quality of the extracted DNA

The means of DNA yield and absorbance ratios (A260/A280 and A260/A230), which reflect the concentration and purity of DNA, are shown in Table 6; Fig. 1, along with statistical comparisons. According to the DNA yield statistics, the combined method (IPF extraction method) produced the highest DNA amounts from most samples, with the non-commercial method (MC extraction method) yielding the second-largest amounts. While the difference between the two approaches was not statistically significant, both resulted in significantly higher DNA yield compared to the commercial methods (PG and MPG) created protocols ($p < 0.05$).

Based on the 260/280 nm ratio findings, the IPF extraction method produced high-quality DNA. If neglecting the reliability of purity caused by DNA concentrations below 20 ng/μl, the PG extraction method can yield DNA purity comparable to that of IPF. In comparison, the other techniques yield DNA of lower purity. The A260/A230 ratios were obtained to assess contamination levels from salts, peptides, and polysaccharides. The results presented in Table 6; Fig. 1 further validate the effectiveness of the IPF extraction method, showing the highest values. Other methods were less effective at removing contaminating compounds.

Gel electrophoresis of the amplified DNA extraction products

Crude DNA was evaluated for its suitability in PCR applications using particular primers ITS2 F94/R and then analyzed using agarose gel electrophoresis (Fig. 2). Overall, the intense amplicons were detected in samples C1 and C2 (lanes 1 to 8), then in samples C3 and C4 (lanes 9 to 16), with sample C5 (lanes 17 to 20) being the lowest in the list. Gel electrophoresis showed that the IPF extraction procedure effectively produced high-quality PCR results from four Chestnut rose juice samples (lanes 4, 8, 12, and 16). There is weak smearing due to excessive loading or high concentration of crude DNA extract. However, the PCR amplicons still displayed higher image intensities for C2-C4 samples than other extraction methods, suggesting the superior quality of the DNA extracts, which aligns with the DNA yields and quality obtained (Table 6; Fig. 1). The gel image of DNA products from the other three procedures exhibited varying quality across distinct samples. Procedure PG (lane 2, 6, 10 and 14) yielded DNA products of comparable quality to procedure IPF, but the visible DNA bands were more vague and less distinct than sample C3 (lane 11). In addition, DNA extracted using the MC and MPG methods

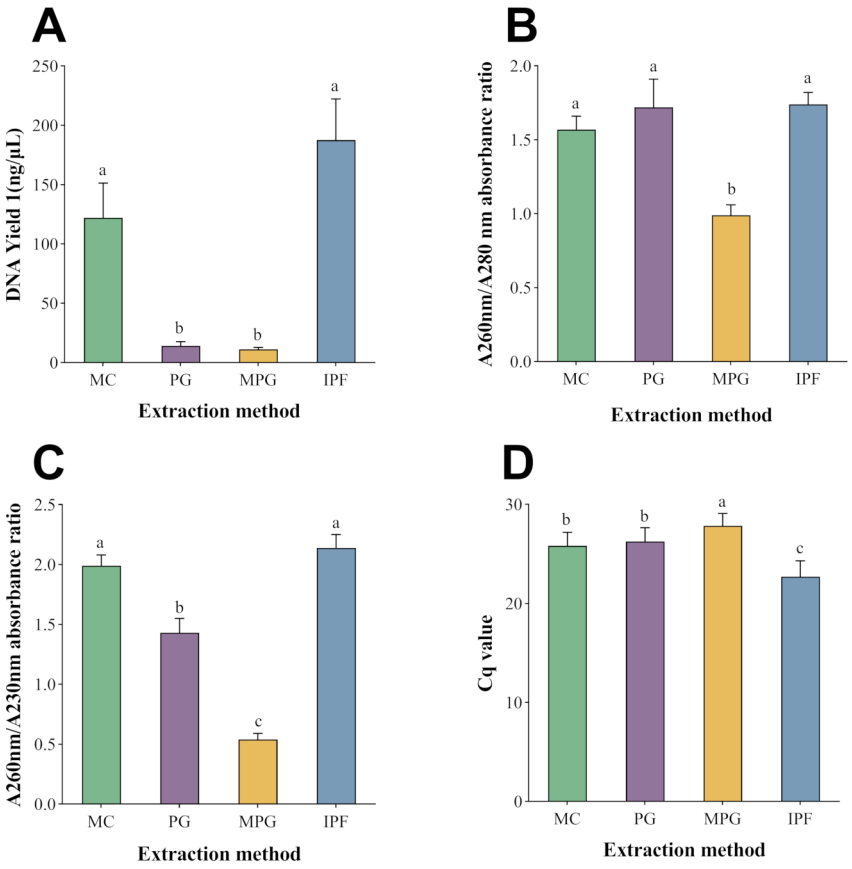

Fig. 1 DNA yield, purity, and Cq value obtained from different sample groups in triplicate using the different extraction methods. Standard deviations from at least three separate experiments are represented by error bars in the column charts. **A**: Chart showing DNA yields obtained for all extraction methods evaluated. **B**: Chart showing absorbance ratios at 260/280 for all extraction methods assessed. **C**: Chart showing absorbance ratios at 260/230 for all evaluated extraction techniques. **D**: Real-time PCR analysis showing ITS2(F94-R) real-time PCR parameters (Cq values) for all extraction techniques examined. [a, b,c, d] Comparison of the values in each column: Suggested changes: Values with different superscripts are significantly different according to the Bonferroni test, $p < 0.05$

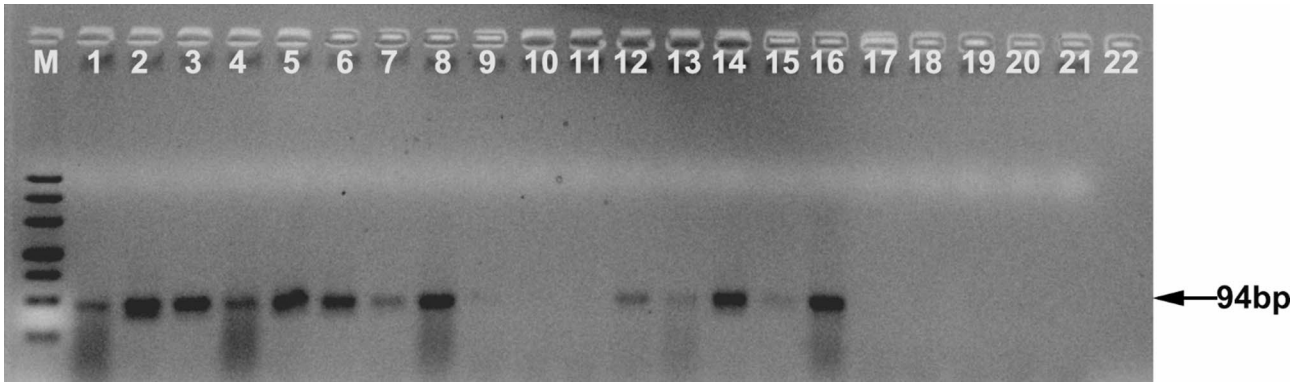

Fig. 2 Representative results from gel electrophoresis analysis of ITS2 gene from five different Chestnut rose samples extracted by four methods. C1 Sample Group (lanes 1-4), C2 Sample Group (lanes 5-8), C3 Sample Group (lanes 9-12), C4 Sample Group (lanes 13-16), C5 Sample Group (lanes 17-20), Blank (lanes 21-22); MC (lane 1, 5, 9, 13, 17), PG (lane 2, 6, 10, 14, 18), MPG (lane 3, 7, 11, 15, 19), IPF (lane 4, 8, 12, 16, 20), (MC: Modified CTAB method, PG: Plant Genomic DNA Kit, MPG: Magnetic Plant Genomic DNA Kit, IPF: isopropyl alcohol precipitation combined with Processed Food DNA Extraction Kit); M: DL500 DNA Marker

yielded relatively satisfactory amplification results in the small sample sizes of C1 and C2. Finally, sample C5 is a less concentrated product with a limited amount of raw material, and the gel image did not reveal clear and distinct bands for all the extraction procedures, so validation using real-time PCR analysis is necessary.

Real-time PCR of the extracted DNA

The real-time PCR analysis findings for four extraction methods from five samples are presented in Supplement Fig. 1. Surprisingly, DNA amplification was achieved for all samples utilizing all four techniques. Lower Cq values are preferable since they indicate higher levels of amplifiable DNA. The Cq analysis conducted on the positive real-time PCR amplifications revealed that Chestnut rose juice samples (C1 to C4) generally exhibited lower values than less concentrated beverage items, such as sample C5. We used the general linear model analysis to evaluate the significance of variations among the real-time PCR Cq values acquired using the four procedures (Table 6; Fig. 1). The IPF extraction method yielded the lowest average Cq value, which was significantly superior to the other extraction procedures ($p < 0.05$). Additionally, no statistically significant changes were determined between the MC and PG extraction methods. It was predicted that the MPG extraction method had the worst performance among all the procedures in the present analysis, which is consistent with the preceding description.

Economic, labor, and time assessments of the extracted DNA

An analysis was also conducted to compare the various DNA extraction approaches' material costs, labor requirements, and throughput time (Table 5). The amount of manual labor needed to extract DNA was virtually identical for all four protocols, and each protocol was straightforward and uncomplicated to adhere to. When each extraction method exclusively focuses on extracting one sample within a specific time frame, DNA extraction times varied from 56 to 217 min across different methods. The commercial methods (PG and MPG extraction procedures) were the shortest, taking 56 min. The MC and IPF extraction processes utilized the isopropyl alcohol precipitation protocol, resulting in DNA extraction times of 163 and 217 min, respectively. Finally substantial differences were observed in material costs when comparing the strategies utilized in this investigation. As anticipated, the costly techniques were the MPG extraction method, followed by the PG and IPF extraction methods. By contrast, The MC extraction method was the most cost-effective, priced at 3.53¥per sample.

Evaluation of the degree of DNA degradation

The maximum size of PCR products that could be amplified indicated the extent of DNA degradation caused by processing. The specific primer pairs for the ITS2 gene successfully amplified fragments of 94, 186, 301, 449, and 500 base pairs (Table 3). Figure 3 displays the largest amplicons obtained from analyzing DNA recovered from marketed Chestnut rose juices and beverages using the IPF extraction methods. Generally, certain samples in the amplification analysis of fragments smaller than 186 bp exhibited comparable amplification curves to the control. However, all samples in the amplification analysis of fragments larger than 301 bp displayed higher Cq values, which were substantially greater than the control values. Additionally, the findings also indicate that all samples yielded a 94-base pair amplicon from the extracted DNA, by contrast, a 500 bp fragment was only found from DNA extracted in specific samples C2 and C4, suggesting that the DNA integrity was influenced by the processing complexity of different manufacturers.

Discussion

The extraction of DNA from food products is a significant accomplishment, especially when considering the various forms of industrial processing they have undergone. Utilizing a single dependable technique for DNA extractions and PCR amplification along the entire juice and beverage supply chain holds potential benefits in several scenarios, such as industrial quality control, for identifying deceit and adulteration. Enhancing national and international legislation regarding the trading and selling of dairy products will also be advantageous. This current investigation conducted a comparative analysis of four extraction methods to establish a foundation for making informed judgments on choosing an appropriate extraction method for a particular sample.

Determination of the appropriate extraction method and selection of samples to be used

After evaluating various DNA extraction procedures for the highly processed samples, we found that the separation and purification stage is essential for efficient DNA extraction. This was further validated by additional research [28]. Therefore, we chose four distinct extraction methods that offer various cell lysis treatments, including chemical, enzymatic, and/or mechanical approaches.

The CTAB-based method was used for this investigation due to the inclusion of CTAB, a positively charged surfactant, in the DNA extraction buffer, which effectively separates and selectively isolates DNA from histone proteins [40]. It has been widely acknowledged as the most reliable and accurate method. It is commonly employed for extracting DNA from different types of plant materials, particularly in highly processed food samples [41, 42]. The CTAB procedure in our laboratory was performed according to the protocol outlined by Andreas et al. [34] with certain modifications. We employed a combination

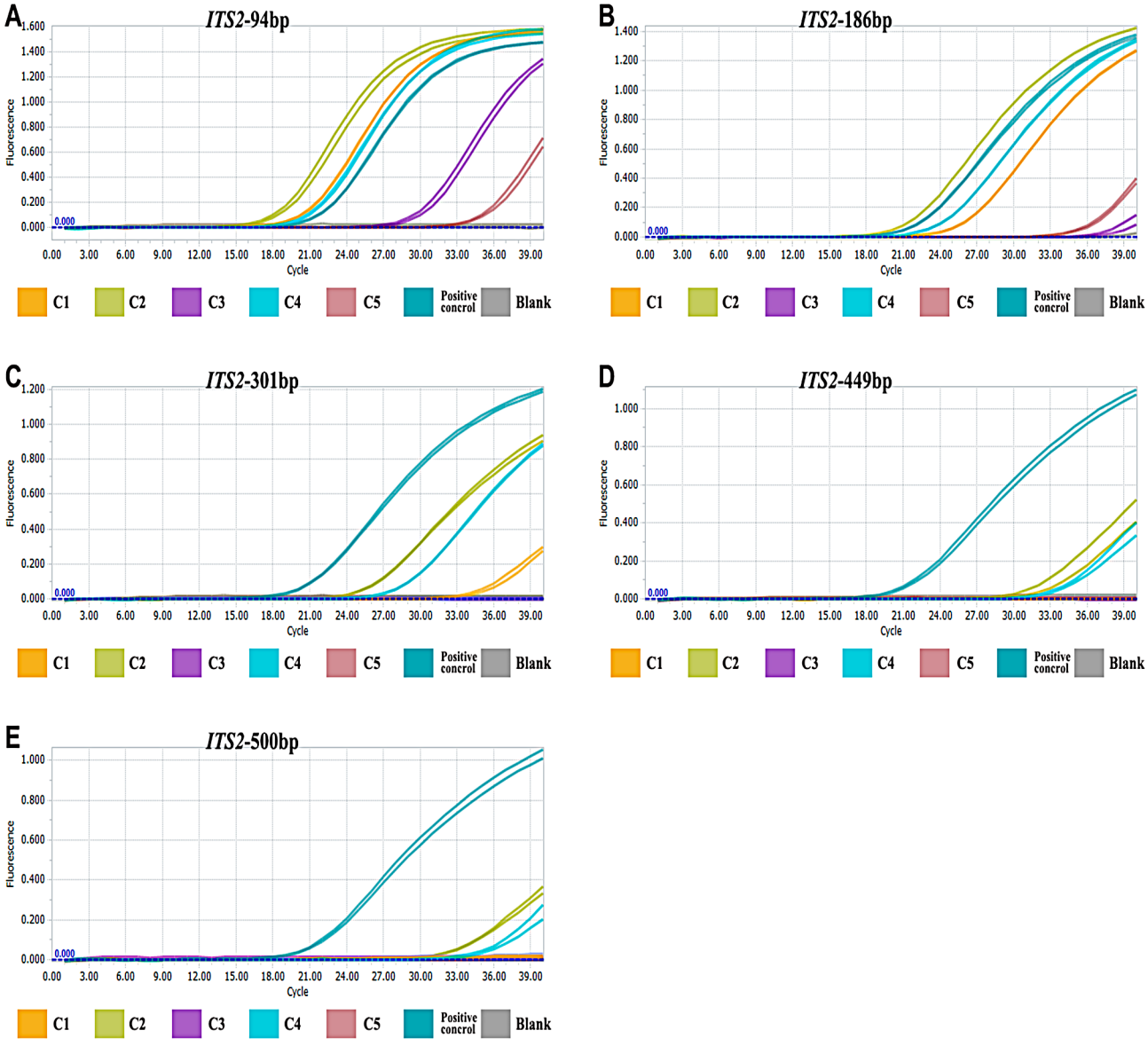

Fig. 3 Real-time fluorescent PCR amplification curves for the ITS2 gene in specific samples with various lengths (**A**: 94 bp, **B**: 186 bp, **C**: 301 bp, **D**: 449 bp, **E**: 500 bp; Positive control: Chestnut rose fruit sample; Blank: Distilled water)

of phenol, chloroform, and isoamyl alcohol (in a ratio of 25:24:1) to eliminate protein contaminants, facilitate the separation of liquid and organic phases, and eliminate the formation of bubbles during the extraction process. In addition, the isopropyl alcohol was subjected to a cold treatment to cause the DNA to separate from the protein, hence minimizing DNA degradation and facilitating their separation. The Plant Genomic DNA Kit was selected as one of the primary spin column-based methods in the current study. Scientists have discovered that spin column-based protocols are suitable for extracting DNA from potato products containing 84 bp DNA fragments [43]. Furthermore, commercial spin column extraction kits are more efficient than standard precipitation methods for extracting DNA from processed foods. The spin column effectively captures fragmented and degraded DNA on the resin membrane, while the chaotropic salts effectively remove PCR inhibitors [44]. We selected the Magnetic Plant Genomic DNA Kit based on the findings of Holden et al.'s [45] study. They confirmed that techniques using DNA binding to a solid matrix, such as silica gel or magnetic particles, yielded superior results for PCR amplifications compared to selective precipitation methods when applied to ground corn. In particular, the Processed Food DNA Extraction Kit is a specialized DNA purification kit produced by the manufacturer TIANGEN specifically for extracting DNA from raw food materials and processed food. It was suggested

because it can effectively eliminate impurities proteins, lipids, and other organic substances. In addition, we implemented alterations to the previous procedure and included the previously mentioned phase of treating with isopropyl alcohol at low temperatures.

The makeup of the sample may provide difficulties for a DNA extraction method. Defining a matrix in food analysis is challenging due to the variability in composition and presence of diverse components that can impact the performance of PCR, even when comparing products made using different processes. The laboratory seldom receives comprehensive compositional data on the products. Hence, we selected five distinct commercial samples to encompass a wide range of potential problems with DNA extraction and a diverse array of matrix complexities. Typically, the provided samples disperse the pulpy components in a liquid medium. Prior to DNA extraction, the samples underwent centrifugal separation treatment to allow dispersed particles from the juice or beverage to settle on the inner surface of the tubes. This process aimed to sufficiently expose all the fibrous particles in the DNA extraction buffer, preventing their dilution. This allowed for optimal contact between the lysate and the cell, enabling the efficient extraction of high-quality DNA from the provided samples. In our study, four distinct extraction methods exhibited varying levels of DNA extraction efficacy. Therefore, we have determined that the effectiveness of DNA extraction is not solely reliant on the characteristics of the sample but also on the specific process used for extracting the DNA. This conclusion was further corroborated by prior research [46].

Evaluation of the extraction method

The assessment of four extraction methods was conducted using spectrophotometer measurements, gel electrophoresis experiments, and real-time PCR Cq values. The three spectrophotometer measurements, namely yield, ratios of 260/280 and 260/230 nm, were preliminarily employed for assessing the quality of DNA. Significant quantities of DNA obtained from the identical sample material are deemed satisfactory, with the ratios falling between 1.7 and 1.9 for 260 nm/280 nm and beyond 2.0 for 260 nm/230 nm being considered acceptable for pure DNA. Reducing the matrix effect by using analogous samples allows us to ascribe the changes in the data to the influence of the extraction procedures. Based on these criteria, only one method, which involved combining isopropyl alcohol precipitation with the Processed Food DNA Extraction Kit (referred to as IPF), proved to be highly effective in the current investigation. The disparities between the IPF extraction method and other methods can be ascribed to the unique buffer system, preprocessing phase, and isopropyl alcohol cold treatment processes. To prevent low efficiency of DNA extraction caused by a pH value that is too low, the samples extracted by the IPF method were pretreated three times with Tris.Cl buffer (pH 8.0), as recommended by the manufacturer. Furthermore, isopropyl alcohol was employed to precipitate DNA by subjecting it to cold treatment for a duration of one hour to maximize the yield of DNA. These measures can be beneficial in obtaining elevated quantities of genomic DNA from all the analyzed products. Regrettably, the chemical factors contributing to the effectiveness of this method cannot be assessed due to the undisclosed nature of the other components in the kit, as provided by the manufacturer.

Regarding the case of highly processed foods, it is possible that significant gene sections cannot be amplified due to structural damage to the DNA. Visualizing the crude DNA on agarose gel alone may lead to incorrect conclusions when assessing the DNA integrity in this particular situation. Therefore, comparing the level of amplifiable endogenous genes among many extracts from the same sample remains a valuable strategy for evaluating extraction techniques. Nevertheless, the level of amplification determined using this method is affected not only by the amount of DNA obtained in the extract but also by the quality of the template DNA. In this context, in contrast to agarose gel visualization of conventional PCR products, real-time PCR demonstrated a distinct signal in all isolated DNA samples, even the less concentrated sample C5. This indicated a remarkable sensitivity, specificity, and dependability level, which aligns with earlier research findings [47–49]. The Cq values obtained from real-time PCR directly indicate the suitability of the extracted sample for molecular investigation. Nevertheless, the Cq values were significantly impacted by PCR inhibitors that were present in the samples and were co-extracted during the various DNA extraction techniques. These inhibitors might potentially disrupt the real-time PCR amplification process, leading to a potential delay in the Cq values or even a full failure of the reaction.

According to real-time PCR Cq measurements, most attention was focused on the results of method IPF, which gave more positive results and made evaluating the different extraction procedures easier. The IPF method was also easily scalable, which may be an important advantage for a difficult matrix such as beverages. Due to the absence of literature references about this method, we could not carry out a comparison analysis. Compared to the IPF extraction method, the noncommercial MC method, specifically the modified CTAB-based approach, yielded higher quantities of DNA in the extracted samples. However, the real-time PCR performance of these samples was relatively poor, which may indicate the presence of inhibitors. The inhibitors can come from studied substances, such as proteins, polysaccharides, lipids,

and polyphenols [50–52]. In addition, the chemicals utilized in the DNA extraction process, including CTAB, EDTA, phenol, chloroform, ethanol, and isopropanol, have the potential to hinder the PCR reaction by inhibiting the Taq polymerase [34, 53–55]. Furthermore, many researches indicated that an extensive array of salts, sugars, and other substances commonly employed in buffer solutions can further diminish the performance of PCR [54, 56, 57]. To address this issue, some researchers have utilized post-DNA extraction methods to enhance the purity of DNA. The methods encompass enzymatic treatments [58], solvent precipitation, and chromatographic or electrophoretic separation [59]. Further purification leads to more effective elimination of PCR inhibitors, resulting in a greater yield of amplification products, assuming that the initial quantity of target DNA is adequately high. Our strategy was not adhered to due to the potential loss of DNA during the purification process, which could destroy samples containing tiny amounts of DNA, such as beverages. It is worth mentioning that the Plant Genomic DNA Kit (abbreviated as PG) successfully removed PCR inhibitors, as described previously. However, this occurred at the cost of reduced yield, since a significant amount of DNA was lost during the column purification process. Consequently, the overall sensitivity of the study was compromised. Evidently, the outcome was confirmed by the observation that the PG approach consistently yielded higher Cq values than the noncommercial MC method. While statistical significance was not achieved (Table 6; Fig. 1), the Cq value disparities between the commercial PG and noncommercial MC methods are still deemed substantial. This is because a one-cycle variation in the Cq value corresponds to doubling the DNA quantity [39]. This finding also indicated that the total amount of DNA obtained was the primary factor affecting the level of detectable endogenous genes in extracts of highly processed foods, which is consistent with a prior study [43]. Finally, it is worth noting the commercial method called Magnetic Plant Genomic DNA Kit (MPG), which, despite yielding DNA with low concentrations and less than ideal purity compared to other methods, still allowed for clear amplification curves with real-time PCR for all the samples in the experiment (Supplement Fig. 1). Researchers have successfully employed this magnetic particle-based technique to isolate DNA from ground corn and maize foodstuffs samples for real-time PCR analysis [45, 60]. The suboptimal performance seen in our study may be attributable to the specific food matrix and product variations from different manufacturers.

Economic evaluation and the assignability across the four procedures

In the field of analytical molecular biology, the process of extracting nucleic acids generally involves making a compromise between achieving high quality and minimizing expenses. Procedures that result in fewer expenses for analyzing the substance generally have worse quality, which might negatively affect the analysis. On the other hand, procedures that produce a highly qualitative analyte often do so at the cost of the economy. While the two commercial kits proved to be the fastest and simplest to perform, the overall expenses per sample were quite high. The reason for this could potentially be explained by the utilization of nucleic acid-binding resins or magnetic particles in these methods. Furthermore, the yield or purity of the extracted DNA using these two methods exhibited poor performance, posing a significant obstacle for samples containing low DNA yields. Compared with other methods, the noncommercial MC method was the most cost-effective regarding supplies. However, it was also time-consuming as it included many transfers between tubes. Transferring samples in a diagnostic environment can lead to a higher risk of cross-contamination. Indeed, we also acknowledged the fact that this approach yielded substantially higher amounts of DNA from all the provided samples. Regarding the combined IPF approach, the processing time is mostly dedicated to sample preparation and cold treatment using isopropyl alcohol to precipitate DNA, resulting in the longest duration. Nevertheless, the entire procedure is straightforward and effortless to operate. Also, the problem of prolonged processing time can be resolved by concurrently processing numerous samples during idle periods. Likewise, we also noticed that while the approach cost was about four times higher than the noncommercial MC method, it fell within the price range of PG and MPG in the commercial kit, positioning it at a reasonable level. Regardless, this method demonstrated outstanding performance in extracting DNA from Chestnut rose juices and beverages. It could be readily expanded to handle more difficult samples, particularly those from highly processed foods with a very low pH value.

Considering the factors mentioned above, we needed to evaluate the assignability of the extraction techniques to various samples of juices and beverages. Due to the enormous variation in the composition and diversity of highly processed food samples, we should have focused on developing improved DNA extraction methods specifically for juices and beverages. This is particularly important for samples with higher levels of acidity. In summary, we established an order for the assignability of the four procedures based on the previously mentioned criteria as follows: IPF > MC > PG > MPG. Each of the four extraction processes has distinct benefits and slight drawbacks.

To achieve specific study objectives, it is advisable to carefully choose the necessary extraction process. If a cost-effective and productive approach is needed specifically for qualitative research, the MC technique would be the preferable choice. In contrast, the IPF approach is appropriate for extracting superior quality DNA, which is essential for precise quantitative analysis.

Evaluation of DNA degradation in chestnut rose juices or beverages

Due to increasing customer demand and technological advancements, various types of processed food products have gained more popularity than fresh produce. This case also contributes to the traceability of food goods, which is crucial for ensuring public health and promoting fair trade. Fraud detection analyses' effectiveness depends on the quality, yield, and degradation level [61], with DNA degradation being a significant issue that can lead to interruptions in species identification and food traceability. HRNČÍROVÁ et al. [41] investigated the impact of technological treatment on DNA degradation in specific plant-based food matrices. The researchers observed a degradation in the integrity of heated DNA that corresponded to the exposure time. The quantity of DNA extracted from a food matrix was influenced by factors such as addition, matrix particle size, and time-dependent thermal treatment. PEANO et al. [62] found the DNA obtained from cracker, taco, tofu, and polenta samples had undergone significant degradation compared to the DNA obtained from corn and soy flours. In addition, the quantity of whole DNA that could be extracted from the more extensively treated products was significantly lower. The size range of DNA fragments retrieved from each sample in our investigation could be affected by the complexity of processing procedures employed by different manufacturers. Based on the findings presented in Fig. 3, it was observed that certain samples exhibited satisfactory performance in the amplification analysis of fragments smaller than 186 bp, whereas all samples showed unsatisfactory results in the amplification analysis of fragments larger than 301 bp. This suggests that the DNA of these samples encountered varying degrees of degradation as a result of different manufacturing processes. Therefore, it impacts the magnification of the largest size of PCR products. Additionally, we also discovered that the overall DNA yield and size of amplifiable DNA fragments obtained from each sample generally exhibited a similar fundamental trend. For instance, Sample C5, a less concentrated beverage product, may have undergone excessive processing operations, resulting in a restricted amplification of DNA fragment size (Fig. 3). Similarly, the total DNA yield obtained was relatively low using a restricted extraction approach (data not shown). Our observation aligns with other research that has shown a decrease in both the quality and amount of DNA obtained from food commodities following food processing, which can have a detrimental impact on the ability to detect or measure food components accurately [43].

Conclusion

In summary, this research demonstrates that the quality and quantity of DNA obtained from Chestnut rose-derived juices and beverages vary depending on the product matrix and extraction process used. The combined IPF method demonstrated exceptional performance across all sample types, affirming its status as the preferred method, presuming that time and cost constraints are not a concern. Alternatively, the inexpensive and high-yielding noncommercial MC approach can be chosen if the quality of extracted DNA is not a major concern. The data acquired from our tests serve as a foundation for making decisions regarding choosing an appropriate extraction technique for a particular sample. Additional investigation should focus on precisely characterizing the constituents concerning their DNA integrity and ability to undergo PCR amplification, as well as developing verified reference materials particular to the matrix.

Abbreviations

PG	Plant Genomic DNA Kit
MPG	Magnetic Plant Genomic DNA Kit
MC	The modified CTAB-based approach
IPF	Isopropyl alcohol precipitation combined with Processed Food DNA Extraction Kit

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

The research conducted in this study was a collaborative effort involving all authors. YR conducted the experiments, contributed to the statistical analysis, and drafted the manuscript. YS and YL were responsible for designing the primers. YM, WZ, and ZX were responsible for designing the techniques and experiments, conducting statistical analysis, and interpreting the results. The work was supported by XH, DY, and JL. YR, YL, and WZ originated the study, conducted data collecting and analysis, contributed to the project's design and coordination, and provided funding for the study. The final manuscript was read and approved by all writers.

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Data availability

Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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