Journal of Food Technology Research
2026 Vol. 13, No. 1, pp. 103-114
ISSN(e): 2312-3796
ISSN(p): 2312-6426
DOI: 10.18488/jftr.v13i1.4800

Molecular authentication of rapeseed in edible oils using double PCR systems

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Article History
Received: 21 October 2025
Revised: 8 January 2026
Accepted: 6 February 2026
Published: 18 February 2026

Keywords
ACCase gene (BnACCg8)
Double PCR
Edible oil
Molecular authentication
Rapeseed (Brassica napus L.).

ABSTRACT

Rapeseed (Brassica napus L.) is one of the world's most important oilseeds, providing edible oil and protein-rich feed. Accurate identification of rapeseed is essential for food and feed authentication, traceability, and safety assessment. This study develops and validates new polymerase chain reaction (PCR) systems for the reliable and specific detection of rapeseed in seeds and processed oils. Primers targeting the rapeseed acetyl-CoA carboxylase (ACCase; BnACCg8) gene were designed, and PCR conditions were optimized following genomic DNA extraction from ground seeds and 700 µL aliquots of edible oils. DNA was isolated using two commercial kits, NucleoSpin Food and Olive Oil DNA Isolation, and PCR products were assessed by agarose gel electrophoresis. Uniplex PCRs demonstrated species specificity, producing 147-bp and 174-bp amplicons only in rapeseed DNA, with no amplification in soybean, sunflower, or maize. PCR bands from oils were weak or absent. Implementing a double-PCR approach increased detection sensitivity in oils by approximately fivefold. Strong, expected-size amplicons were obtained from all oil extracts, confirming reliable detection of rapeseed in both cold-pressed and refined varieties, regardless of extraction method. This approach offers a sensitive, rapeseed-specific molecular tool for verifying the botanical origin of edible oils. It is suitable for routine authenticity testing and quality control of rapeseed-based products, supporting regulatory compliance and reducing mislabeling risks within oil supply chains.

Contribution/Originality: This study introduces novel PCR systems for the precise identification of rapeseed in seeds and processed oils. It is the first to detect rapeseed DNA in 700 µL of edible cold-pressed and refined oils. The research enhances authenticity testing and quality control of oil products.

1. INTRODUCTION

Rapeseed (*Brassica napus L*) is a significant oilseed crop with importance in the global food industry. Its primary uses include the oil extracted from seeds and the residue remaining after oil extraction (cake/meal). The main product for human consumption is rapeseed oil, valued for its healthy fats, while the protein-rich rapeseed meal is used as animal feed (Food and Agriculture Organization of the United Nations (FAO), 2023; Gu, Guan, Jiao, Liu, & Hong, 2024). Although palm and soybean oils dominate the global vegetable-oil market, rapeseed oil still holds a significant

share and maintains commercial value (U.S. Department of Agriculture Foreign Agricultural Service 2025). Confirming the botanical origin of rapeseed in processed oils remains challenging, especially when molecular tools are needed to differentiate it from other oil sources.

The oil pressed from rapeseed, especially from modern low-erucic-acid types such as canola, 00-rapeseed, LEAR cultivars, and canola-quality *Brassica juncea*, is valued for its fatty-acid profile, notably its high levels of oleic acid and α-linolenic acid (Codex Alimentarius Commission, 1999; Lin et al., 2013). These nutritional qualities support its widespread use in cooking and processed foods. Additionally, rapeseed oil is increasingly important as a renewable industrial material, especially in biodiesel and bio-lubricants, contributing to low-carbon technology efforts in Europe and beyond (Osman, Rahman, & Ali, 2024; Suchocki, 2024).

Beyond oil, various other rapeseed-derived products contribute to food systems and value chains. In Japan, young shoots and flower buds ("nanohana" or "nabana") are consumed as seasonal vegetables (Kagawa Prefectural Government, 2025). Rapeseed or canola honey is also a common monofloral honey in many regions (Bratosin et al., 2025; García-Tenesaca et al., 2018). Rapeseed protein isolates and concentrates, evaluated as novel food ingredients and granted GRAS status, are increasingly used in bakery items, dairy alternatives, and other formulated foods due to their functional and nutritional properties (EFSA Panel on Dietetic Products, Nutrition, & Allergies, 2013; U.S. Food and Drug Administration (FDA), 2025a, 2025b). Recent studies have demonstrated their utility in gluten-free baked goods, improving texture and structural stability (Di Lena et al., 2023; Korus, Chmielewska, Witczak, Ziobro, & Juszczak, 2021).

Rapeseed (or canola) is significant in modern crop biotechnology. It is one of the four major genetically modified (GM) crops grown commercially, alongside soybean, maize, and cotton. Most GM varieties are herbicide-tolerant (HT), providing tolerance to glyphosate or glufosinate. These traits simplify weed management and are compatible with reduced-tillage systems, making them appealing to growers. Currently, GM cultivars comprise about a quarter of global rapeseed acreage, especially in major exporting countries. Importantly, oil and meal from GM and non-GM rapeseed are compositionally similar and are used interchangeably in food and feed markets (International Service for the Acquisition of Agri-biotech Applications (ISAAA), 2020).

Due to its economic and nutritional importance, its agronomic role, and its relevance to sustainable development initiatives, rapeseed is frequently the subject of agricultural, biotechnological, and environmental research. It has also become an important matrix in food authenticity studies. Species can be authenticated by molecular, biochemical, and chemical characteristics. DNA molecules are more stable than proteins under stressful food processing conditions. Therefore, DNA-based molecular methods are particularly effective in processed products, where proteins and chemical profiles are severely degraded, but DNA fragments still retain species-specific information. Many authentication efforts now focus on DNA-based assays, as these methods enable species-level identification even when oils have undergone significant processing (Gu et al., 2024; Hellebrand, Nagy, & Mörsel, 1998; Srivastava et al., 2024). The high value of rapeseed oil and the complexity of its international trade create opportunities for economically motivated adulteration. Reliable species verification is essential and urgent for regulatory and commercial oversight (Kanwal, Sun, Tong, & Alhamad, 2024; Zhang, Zhu, Duan, & Zhao, 2024).

Although many analytical techniques exist for testing edible oils, conventional methods often struggle with refined oils. DNA in these matrices is limited, heavily fragmented, and co-extracted with substances like lipids, phenolics, and surfactants that can inhibit PCR, complicating analysis (Bojang, Ki, & Kim, 2021; Debode, Janssen, Marien, & Berben, 2012; He, Zheng, Zhou, & Liu, 2013). These issues decrease amplification efficiency and may cause inconclusive or false-negative results unless extraction and PCR conditions are optimized. To address these challenges, various groups have developed modified PCR strategies, such as improved extraction protocols and short-amplicon or multi-step assays, which can better tolerate degraded DNA and low template quantities (Kutateladze et al., 2024; Testolin & Lain, 2005; Vahdani, Farahani, & Bitaraf, 2024).

For rapeseed, species-specific and GM-targeted assays have been validated mainly on plant tissues or processed foods, demonstrating that DNA-based identification is feasible in many circumstances (Chhalliyil et al., 2020; Kutateladze et al., 2024; Park et al., 2022; Schmidt, Zelman, & Lüthy, 2006; Vahdani et al., 2024). However, only a limited number of studies have attempted direct detection in edible oils. Those that exist often depend on relatively large oil volumes and still report inconsistent DNA yields, especially from highly refined samples (Debode et al., 2012; Park et al., 2022; Testolin & Lain, 2005). Such results illustrate persistent analytical barriers and highlight the need for more reliable approaches tailored to oil matrices.

Previous research indicates that rapeseed DNA can be extracted from various products, but extracting DNA from edible oils remains challenging with inconsistent yields. Early protocols, originally designed for leaf or seed material, are often unsuitable for oils due to abundant inhibitors and poor DNA integrity. In this study, we address these issues by employing an oil-adapted dual polymerase chain reaction (PCR) strategy. This method enhances sensitivity and reduces the effects of inhibitors, providing a more effective way to detect rapeseed DNA traces in both cold-pressed and refined oils.

To close the methodological gap, we designed and optimized two uniplex PCR assays using newly developed *ACCase*-targeting primers for *B. napus*. Based on these uniplex methods, two dual-PCR systems were developed to improve analytical sensitivity. These assays were applied to DNA from ground seeds and representative commercial oil types. Using short amplicons and a two-step PCR workflow, we detected rapeseed DNA from oil volumes as small as 700 µL. To our knowledge, analyzing such small oil amounts has not been reliably demonstrated previously. The resulting method offers a valuable tool for laboratories aiming to routinely authenticate rapeseed species in oil matrices. This supports efforts to maintain product quality in the edible oil supply chain.

This study was guided by three main questions. First, can the newly designed *ACCase*-based primers effectively amplify B. napus DNA in oil matrices where DNA is scarce and degraded? Second, does the double-PCR approach provide a clear sensitivity improvement over standard uniplex PCR? Third, is the system suitable for small-volume, practical authentication of both cold-pressed and refined rapeseed oils?

The paper is organized into sections. The Methods section details DNA extraction, primer design, and the development of uniplex and dual-PCR protocols. The Results section compares findings from seeds and oils, focusing on sensitivity differences. The Discussion interprets these results and their relevance for routine authenticity testing. The Conclusions summarize key contributions and suggest future directions for DNA-based identification of edible oils.

2. MATERIALS AND METHODS

This study employed a DNA-based analytical procedure with several sequential steps: selecting the rapeseed-specific *acetyl-CoA carboxylase (ACCase)* gene (1); designing and synthesizing PCR primers for the *ACCase* gene (2); extracting genomic DNA from rapeseed seeds and oils (3); developing and optimizing uniplex PCR systems for seed DNA detection (4); and creating double PCR methods to identify rapeseed DNA in oils (5). Unlike previous methods that often used large sample volumes or single-round amplification optimized for plant tissues rather than oils, this approach combines oil-adapted extraction, short *ACCase* targets, and a double-PCR technique to improve the detection efficiency of rapeseed in small amounts of cold-pressed and refined oils.

2.1. Plant and Oil Materials

Seeds of soybean (*Glycine max*), maize (*Zea mays*), and sunflower (*Helianthus annuus L.*), along with cold-pressed and refined rapeseed oils, were purchased from supermarkets in Tbilisi, Georgia. The rapeseed seeds were supplied by the Georgian biodiesel company "Biodiesel Georgia LLC". Seeds were milled into a homogeneous flour using an electric grinder (Tube Mill 100 control, IKA, Staufen, Germany). All oils were stored at 4 °C until analysis.

2.2. Genomic DNA Extraction

Genomic DNA was extracted from 100 mg of ground rapeseed, sunflower, maize, and soybean seeds using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For oils, 700 µL aliquots were processed with two commercial kits, the NucleoSpin Food Kit (MACHEREY-NAGEL, Düren, Germany) and the Olive Oil DNA Isolation Kit (Norgen Biotek Corp., Thorold, ON, Canada) following the manufacturers' instructions.

2.3. PCR Analysis

Based on a comprehensive review of the literature and available GenBank records, the *acetyl-CoA carboxylase (ACCase)* gene of rapeseed (*Brassica napus* L.), corresponding to the BnACCg8 locus (GenBank accession X77576.1), was selected as the species-specific molecular target for PCR assay development (Debode et al., 2012; Schmidt et al., 2006; Wu, Zhang, Wu, Cao, & Lu, 2010). The sequence information was retrieved from the GenBank Nucleotide Database (https://www.ncbi.nlm.nih.gov/nucleotide; accessed 5 September 2024).

Oligonucleotide primers specific to B. napus ACCase were designed using Primer-BLAST (Ye et al., 2012) from NCBI and the PrimerQuest™ Tool (Integrated DNA Technologies; https://eu.idtdna.com/PrimerQuest; accessed 12 March 2024). The primer sequences (5′→3′) generate short, highly specific amplicons suitable for processed food matrices.

- Rapeacc147f: AGCAACTTGCGGAGATTGGG.
- Rapeacc147r: CACCTGCAACGCACATATTCA.
- Rapeacc174f: AGCGAGTTGAGAGCACAGAAA.
- Rapeacc174r: ACCTGCAACGCACATATTCAC.

In addition, two eukaryote-specific primer sets targeting the 18S rRNA gene were adopted from the literature to evaluate the amplifiability of DNA recovered from oil matrices (Duan, Pi, Li, & Jiang, 2021; Zhang, Cai, Guan, & Chen, 2015). The primer pair 18S-140f/18S-140r was sourced from Zhang et al. (2015), and 18S-167f/18S-167r from Duan et al. (2021). These multi-copy nuclear targets produce short amplicons (140 bp and 167 bp), suitable for fragmented DNA in processed edible oils, serving as internal controls for method development and matrix suitability assessment. The sequences are provided below:

- 18S-140f: TCTGCCCTATCAACTTTCGATGGTA.
- 18S-140r: AATTTGCGCGCCTGCTGCCTTCCTT.
- 18S-167f: GCAAGACCGAAACTCAAAGGA.
- 18S-167r: ACGACAGCCATGCAGCACC.

All oligonucleotides were synthesized and HPLC-purified by Integrated DNA Technologies (IDT, Coralville, IA, USA).

The PCR conditions for the newly designed ACCase primer pairs were systematically optimized through empirical screening of key reaction parameters.

The optimization process involved testing several key PCR parameters to identify conditions that provided both strong amplification and high specificity. Magnesium chloride was varied from 1.0 to 6.0 mM to find the balance between polymerase activity and selective primer binding. Primer concentrations were adjusted between 0.10 and 1.50 µM to reduce nonspecific products while preserving sufficient signal. An annealing temperature gradient from 52 to 68 °C was used to determine the most selective hybridization temperature, and extension times were explored within a 20–90 s range to match the expected amplicon lengths without promoting undesired amplification. The number of cycles was also evaluated, from 30 to 60, to identify the lowest cycle count that still produced consistent, interpretable bands. Final PCR conditions were chosen based on band clarity, absence of off-target amplification, and reproducibility across independent DNA extractions and repeated runs. These optimized parameters were applied uniformly to all uniplex and double-PCR assays.

After optimization, PCR amplifications were performed in a total reaction volume of 25 µL, containing 1× Taq buffer (1.5 mM $MgCl_2$), 0.2 mM of each dNTP (Deoxynucleotide Mix), 1.25 U Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA), and 0.5 µM of each primer. Template DNA was added as 1 µL of seed DNA or 2 µL of oil DNA extract. For the 18S systems, primer concentration was adjusted to 0.4 µM to optimize amplification balance. The double-PCR approach used 2 µL of the first-round PCR product as the template for the second amplification. Sterile nuclease-free water served as a no-template control in every assay.

PCR reactions were performed on a Techne TC-412 thermal cycler (Techne, Minneapolis, MN, USA). Cycling parameters were as follows.

- Uniplex PCR (*ACCase* primers): 95 °C for 4 min; 35 cycles of 95 °C for 40 s, 58 °C for 45 s, 72 °C for 45 s; final extension 72 °C for 5 min.
- Double PCR: 95 °C for 4 min; 35 cycles of 95 °C for 35 s, 58 °C for 40 s, 72 °C for 40 s; final extension 72 °C for 5 min.
- 18S rRNA systems: 95 °C for 4 min; 35 cycles of 95 °C for 40 s, 56 °C for 45 s, 72 °C for 45 s; final extension 72 °C for 5 min.

PCR products were separated on 2% agarose gels prepared with ethidium bromide (1 µg/mL) using a VWR electrophoresis unit (VWR International, Radnor, PA, USA). After electrophoresis, bands were visualized under UV light and documented with a PhotoDoc-It imaging system (UVP, Upland, CA, USA). These gel profiles enabled evaluation of amplicon size, product yield, and amplification specificity, confirming the reliability and overall performance of the optimized PCR systems.

2.4. Statistical Analysis

To evaluate assay reliability, repeatability, and sensitivity across independent replicates were assessed. Agarose gel band intensities were quantified using ImageJ 1.54r (NIH, USA). The integrated density values were measured within consistently defined regions of interest for each lane. Each PCR assay was performed in triplicate. The mean intensity and standard deviation were calculated to assess repeatability. To compare the performance of uniplex and double PCR assays, the study evaluated the consistency of band appearance across replicates. These measurements provided a direct indication of the assay's reliability and relative sensitivity.

3. RESULTS AND DISCUSSION

3.1. Identification of Effective PCR Primers for Rapeseed Detection

For molecular identification of species, PCR-based assays target genomic regions that are unique and characteristic of a given species. This allows precise differentiation from closely related taxonomic groups. For rapeseed (*Brassica napus L.*), several such markers have been identified and successfully used in diagnostic PCR systems. These include the *acetyl-CoA carboxylase (ACCase)* gene, a set of *orphan* genes (OGs), and various *Brassica-specific* genes (BSGs). They are the most relevant genomic targets for species-level identification.

The *acetyl-CoA carboxylase (ACCase)* gene encodes a key enzyme in fatty-acid biosynthesis, converting acetyl-CoA to malonyl-CoA. In *Brassica napus*, the ACCg8 locus is mainly present as a single copy and contains conserved functional regions alongside species-specific polymorphisms that distinguish B. napus from other Brassica species and unrelated plants. Its demonstrated specificity showed no amplification in B. rapa, B. oleracea, Arabidopsis thaliana, maize, or soybean. Consequently, the *ACCase* gene is widely recognized and used as a reliable endogenous marker for molecular identification, quantification, and authenticity testing of rapeseed in food and feed matrices (Debode et al., 2012; Schmidt et al., 2006; Wu et al., 2010).

In this study, the *ACCg8* gene of *B. napus* was identified as the optimal diagnostic target for sensitive PCR detection of rapeseed. Using the reference sequence (GenBank accession X77576.1), multiple novel primer pairs were designed and evaluated for amplification efficiency and specificity through uniplex PCR assays. Genomic DNA from

ground rapeseed seeds served as the positive control, while DNA from soybean (*Glycine max*), sunflower (*Helianthus annuus*), and maize (*Zea mays*) were used as non-target controls to test species discrimination capacity.

The PCR amplification profiles are shown in Figure 1, which depicts agarose gel electrophoresis of products generated with primer pairs rapeacc147f/rapeacc147r (lanes 1–5) and rapeacc174f/rapeacc174r (lanes 6–10).

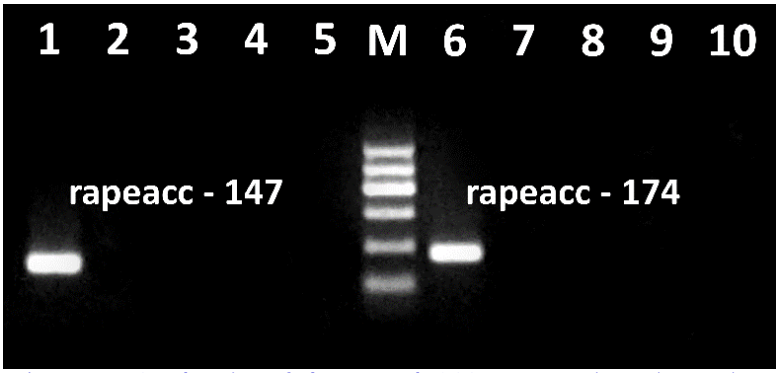

Figure 1. PCR detection of the rapeseed ACCase gene using primer pairs rapeacc147f/rapeacc147r (lanes 1–5; expected amplicon 147 bp) and rapeacc174f/rapeacc174r (lanes 6–10; expected amplicon 174 bp). Samples: rapeseed seeds (lanes 1, 6); soybean seeds (lanes 2, 7); sunflower seeds (lanes 3, 8); maize seeds (lanes 4, 9); water, no-template negative control (NC) (lanes 5, 10).

Both primer sets, rapeacc147f/rapeacc147r and rapeacc174f/rapeacc174r, successfully amplified the expected single fragments of 147 bp and 174 bp, respectively, exclusively from rapeseed genomic DNA (Figure 1, lanes 1, 6). No amplification products appeared with DNA from soybean, sunflower, or maize (Figure 1, lanes 2–4, 7–9), and no-template water controls showed no signal (Figure 1, lanes 5, 10), confirming assay specificity and absence of contamination.

The results support previous research identifying the BnACCg8 locus as a reliable genetic marker for Brassica napus, as shown by (Debode et al., 2012; Schmidt et al., 2006; Wu et al., 2010). The findings confirm that the newly developed primer pairs enable highly specific and sensitive detection of rapeseed DNA across various sample types. Consequently, both primer sets were chosen for further PCR analysis of cold-pressed and refined rapeseed oils.

3.2. Assessment of the Amplifiability of Oil DNAs

In this study, two major types of rapeseed edible oils, cold-pressed and refined, were examined. These oils are produced on a large industrial scale and are widely used in food production and daily cooking, including salad dressings, baking, and frying. Their molecular traceability and authenticity assessment are of significant scientific and practical importance.

Successful PCR-based detection of plant material in oils relies on obtaining sufficient amounts of amplifiable genomic DNA. Extracting DNA from plant-derived matrices is often challenging because metabolites such as polysaccharides, polyphenols, lipids, and pigments can co-extract with nucleic acids and inhibit enzymatic reactions. Therefore, extraction procedures often need species-specific optimization based on the chemical properties of the matrix. In edible oils, additional challenges include mechanical, chemical, and thermal processing, which cause DNA degradation and fragmentation. This significantly reduces amplification efficiency and complicates PCR detection, requiring tailored methods to improve DNA recovery and detection accuracy (Bojang et al., 2021; Debode et al., 2012; He et al., 2013; Kutateladze et al., 2024; Testolin & Lain, 2005).

Previous studies indicate that detecting minimal residual DNA in edible oils often necessitates preconcentration or enrichment steps, involving processing large oil volumes (10 to 500 ml) to obtain adequate DNA samples for analysis (Bojang et al., 2021; He et al., 2013; Kutateladze et al., 2024; Testolin & Lain, 2005).

These approaches are typically laborious, slow, and expensive, and may not always yield DNA of adequate quality for PCR analysis. Commercial extraction kits have simplified the workflow and improved reproducibility. However, the amount, quality, and purity of DNA isolated can still vary significantly depending on the plant source, oil type, and degree of processing. Therefore, for each edible oil, it is essential to experimentally verify and optimize the DNA isolation procedure to ensure sufficient recovery of intact, amplifiable DNA for downstream molecular assays.

In this study, two commercial extraction kits, NucleoSpin Food (MACHEREY-NAGEL, Germany) and Olive Oil DNA Isolation Kit (Norgen Biotek, Canada), were selected for comparison. Both kits had previously shown good performance in extracting amplifiable DNA from other oil matrices, such as olive and sunflower oils, but had not been validated for rapeseed oil. The oil sample volume was fixed at 700 µL, based on earlier optimization results for sunflower oil DNA extraction (Kutateladze et al., 2024).

The sensitivity of PCR detection is strongly influenced by the target gene's copy number (Debode et al., 2012; Kutateladze et al., 2024). For samples with low DNA concentrations, such as edible oils, PCR assays targeting multi-copy genes (e.g., ribosomal RNA genes) generally yield stronger amplification signals than those targeting single- or low-copy species-specific genes. In oil production, both cold-pressed and refined processes expose seeds to high pressure and temperature, causing extensive DNA degradation and resulting in short, fragmented nucleic acids. Therefore, before testing rapeseed-specific primers, the amplifiability of oil-extracted DNA was evaluated using PCR systems targeting the 18S ribosomal RNA gene, a high-copy nuclear marker used as an internal amplification control (Figure 2).

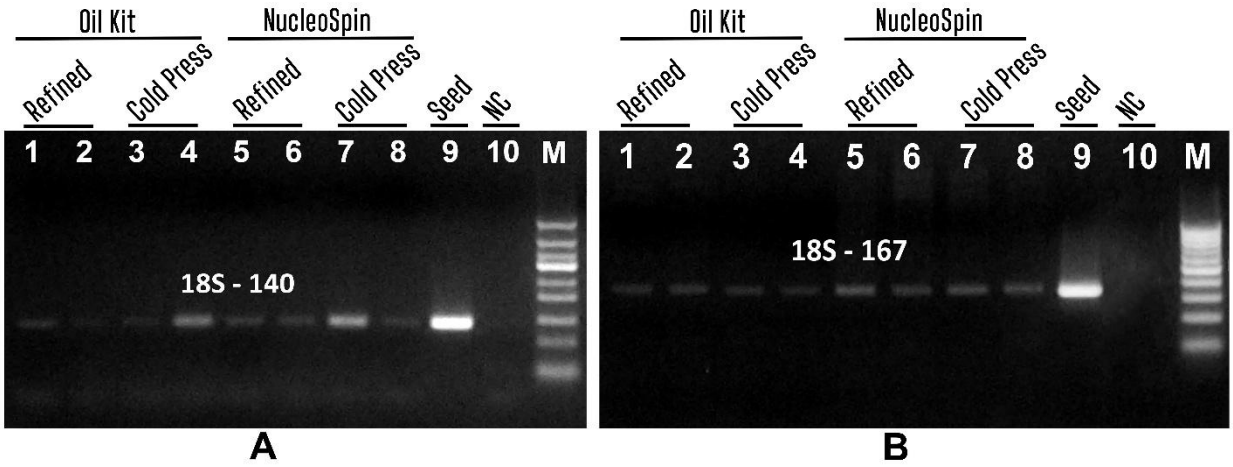

Figure 2. PCR amplification with primers 18S-140f/18S-140r (A) and 18S-167f/18S-167r (B) of DNAs extracted from oils. DNA was isolated using the Olive Oil DNA Isolation Kit (lanes 1–4) or NucleoSpin Food Kit (lanes 5–8). Samples include refined rapeseed oil (lanes 1–2, 5–6), cold-pressed rapeseed oil (lanes 3–4, 7–8), rapeseed seed DNA (lane 9), and water, no-template negative control (lane 10). M, molecular size standard: Qiagen GelPilot 50 bp Ladder.

This study utilized two eukaryote-specific PCR systems to assess the amplifiability of genomic DNA extracted from rapeseed oil samples. These systems target the multi-copy 18S ribosomal RNA (rRNA) gene, a highly conserved nuclear marker present in all eukaryotic genomes, often used as an internal control for DNA quality and amplification potential. The primer pairs 18S-140f/18S-140r and 18S-167f/18S-167r amplify fragments of 140 bp (Zhang et al., 2015) and 167 bp (Duan et al., 2021), suitable for analyzing fragmented DNA from processed foods like edible oils. As shown in Figure 2, both primer pairs produced amplification products of expected sizes in all samples. Clear, intense bands corresponding to 140 bp and 167 bp fragments were observed in seed DNA reactions (Figure 2A, B, lane 9), confirming the high sensitivity and efficiency of these PCR systems under the chosen conditions.

All DNAs from the oil samples produced visible PCR amplicons of expected sizes: 140 bp with primers 18S-140f/18S-140r (Figure 2A, lanes 1–8) and 167 bp with primers 18S-167f/18S-167r (Figure 2B, lanes 1–8). This shows that DNAs from both cold-pressed and refined rapeseed oils maintained sufficient integrity and quantity for successful

amplification. Notably, PCR bands from oil samples were weaker than those from seeds, indicating lower yield and greater degradation of oil DNAs in treated matrices. No amplification occurred in the negative water control (Figure 2A, B, lane 10), confirming assay specificity and absence of contamination.

The successful amplification of the 18S rRNA gene from DNA samples obtained using two DNA isolation methods confirms that intact, amplifiable DNA fragments were present in both types of oil extracts. These results verify that both extraction methods used in this study (NucleoSpin Food Kit and Olive Oil DNA Isolation Kit) can produce PCR-compatible DNA extracts from cold-pressed and refined oils. The absence of amplified product in negative water controls indicates the workflow's purity and the assay's overall reliability. Collectively, these findings demonstrate that DNA obtained from the analyzed oil samples is reliably amplifiable and suitable for subsequent species-specific PCR detection of rapeseed.

3.3. Development of Double PCRs for Rapeseed Detection in Edible Oils

After confirming the amplifiability of the extracted oil DNAs using the 18S rRNA marker system, the next phase focused on developing and validating species-specific PCR assays for reliable rapeseed DNA identification in edible oils. Following successful primer specificity verification on plant-derived DNA, primer pairs rapeacc147f/rapeacc147r and rapeacc174f/rapeacc174r, targeting the *B. napus acetyl-CoA carboxylase (ACCase)* gene, were evaluated on DNA directly isolated from oil matrices. Both cold-pressed and refined rapeseed oils, representing major commercial forms, were analyzed.

DNA extracts from both the NucleoSpin Food Kit and Olive Oil DNA Isolation Kit yielded weak or near-threshold amplicons in conventional uniplex PCR, with some samples showing barely detectable signals (Figure 3A, C, lanes 1–8). These results suggest that single-round amplification lacks the sensitivity required for routine species identification in processed oils, likely due to low yield and degraded DNA resulting from industrial refining. To address this, an improved double-PCR (nested-PCR-like) method was introduced to enhance amplification efficiency and detection sensitivity (Schmidt et al., 2006; Vahdani et al., 2024).

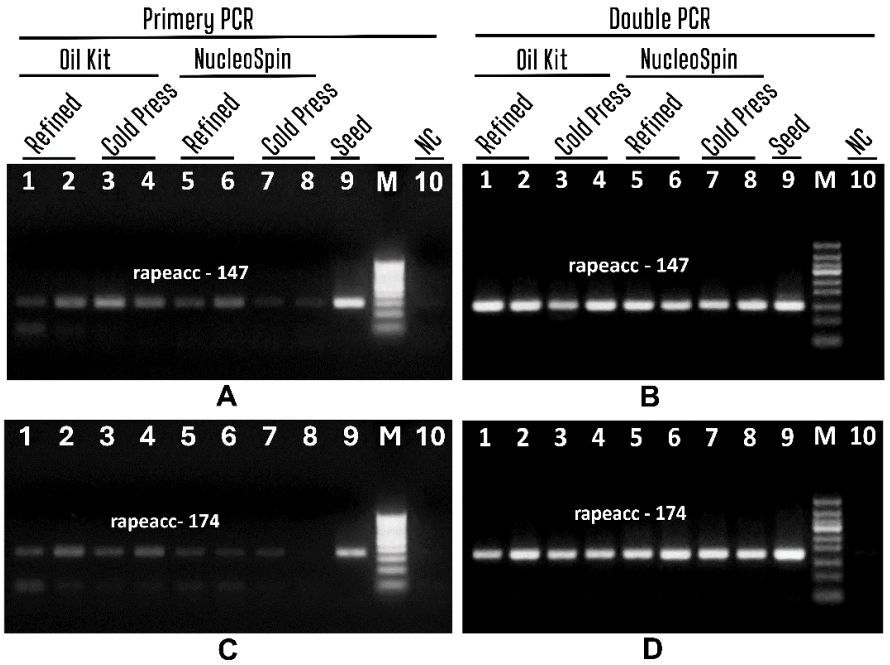

Figure 3. Uniplex (A, C) and double-PCR (B, D) detection of the rapeseed *ACCase* gene using primer pairs rapeacc147f/rapeacc147r (A, B; expected 147 bp) and rapeacc174f/rapeacc174r (C, D; expected 174 bp) from DNA extracted directly from oils. DNA was isolated with the Olive Oil DNA Isolation Kit (lanes 1–4) or NucleoSpin Food Kit (lanes 5–8). Samples: refined rapeseed oil (lanes 1–2, 5–6); cold-pressed rapeseed oil (lanes 3–4, 7–8); rapeseed seed DNA (lane 9); water, no-template negative control (NC) (lane 10). M, molecular size standard: Qiagen GelPilot 50 bp Ladder.

The implementation of this double-PCR approach significantly enhanced assay performance. Both systems using rapeacc147f/rapeacc147r and rapeacc174f/rapeacc174r successfully generated the expected 147-bp and 174-bp amplicons across all oil extracts, regardless of extraction method (Figure 3B, D, lanes 1–8). These results confirm the reliable, reproducible detection of rapeseed DNA in cold-pressed and highly refined oil matrices. To our knowledge, this is the first report demonstrating the extraction and successful amplification of rapeseed DNA from edible oils using the NucleoSpin Food and Olive Oil DNA Isolation kits. The findings align with previous studies indicating these commercial kits' suitability for other oilseed matrices such as sunflower oil (Kutateladze et al., 2024). Furthermore, rapeseed seed DNA produced strong, consistent PCR bands under both uniplex and double-PCR conditions (Figure 3A–D, lane 9), confirming the high amplification efficiency of the primers. No-template controls showed no amplification signals (Figure 3A–D, lane 10), ensuring assay specificity and cleanliness.

The analysis of agarose gels using ImageJ revealed significant improvements in signal strength and detection consistency with the double-PCR method. Uniplex PCR produced weaker, more variable bands, many near the detection threshold. Conversely, double-PCR consistently generated strong, well-defined amplicons across all replicates. Quantitative measurements supported these findings: the average band intensity for uniplex reactions with primers rapeacc147f/rapeacc147r was 18,638, while for double PCR it was 72,056, indicating approximately a 3.9-fold increase. Similarly, for primers rapeacc174f/rapeacc174r, uniplex reactions had an average intensity of 12,203, compared to 70,445 for double PCR, a roughly 5.8-fold increase. Overall, these results demonstrate that the double PCR approach significantly enhances detection sensitivity, with a 4–6-fold increase in signal intensity compared to uniplex reactions. All double-PCR reactions produced positive bands even at lower DNA concentrations, underscoring the higher efficiency and reliability of the double-step amplification method under the tested conditions.

Earlier research has shown that detecting plant DNA in edible oils, especially refined products, is technically challenging due to severe DNA degradation and inhibitory compounds (Testolin & Lain, 2005). Reliable amplification was mainly limited to crude oils, with refined samples often requiring larger volumes to obtain detectable PCR products. Similar issues were reported in another study, which noted inconsistent DNA recovery and variable amplification success across commercial oils using conventional PCR methods (Vahdani et al., 2024). In contrast, our results show that the double PCR system developed in this study consistently detected rapeseed DNA from only 700 µL of refined oil, a smaller volume than previously required. This enhanced performance indicates that combining short *ACCase* amplicons with a double amplification step effectively overcomes earlier limitations, offering a more sensitive and robust method for species authentication in highly processed oil matrices.

4. CONCLUSION

This study developed and validated rapeseed-specific PCR assays targeting the *ACCase (BnACCg8)* gene, using primer pairs rapeacc147f/rapeacc147r and rapeacc174f/rapeacc174r. Both primer sets consistently produced the expected 147-bp and 174-bp amplicons exclusively from *Brassica napus* DNA, with no cross-amplification from soybean, sunflower, or maize, and negative no-template controls, confirming high analytical specificity.

The amplifiability of oil-derived DNAs extracted using the NucleoSpin Food Kit and the Olive Oil DNA Isolation Kit was confirmed through two eukaryote-specific PCR systems targeting the multicopy 18S rRNA gene. While *ACCase*-based uniplex PCR produced strong signals from plant DNA, it lacked sufficient sensitivity for routine analysis of oil matrices, where DNA is scarce and often degraded. To overcome this, a double PCR approach was implemented, significantly enhancing detection sensitivity and consistency. Using this method, rapeseed DNA was reliably amplified from as little as 700 µL of both cold-pressed and refined oil. To our knowledge, this is the first demonstration of *B. napus* detection from refined oil in this sample volume using commercially available extraction kits. This protocol offers a practical, efficient solution suitable for routine laboratory workflows. Because it requires only a small amount of oil and standard PCR equipment, it is easily applicable in industrial quality control, commercial food testing laboratories, and regulatory agencies responsible for verifying label claims.

The findings of this study have clear implications for food authenticity testing. The developed *ACCase*-based PCR assays are sensitive and practical tools for confirming the botanical origin of edible oils. They enable reliable detection of rapeseed DNA even in high-quality refined products. By enhancing traceability in the supply chain, this method provides value to routine testing laboratories and regulatory agencies seeking to prevent adulteration and ensure accurate labeling.

Some limitations should be noted. DNA recovery from refined oils remains inherently low and may vary depending on processing conditions. In very stringent refining scenarios, the DNA detected may still be below the threshold, even with the dual PCR approach. The method was only evaluated for rapeseed, and its quantitative capabilities in blended oil adulteration scenarios have not been assessed.

Future studies may explore additional edible oils and blended oils. Incorporating real-time PCR could enhance sensitivity and enable quantification, expanding its industrial applications.

Funding: This research was supported by Shota Rustaveli National Science Foundation of Georgia (SRNSFG) (Grant number: AR-22-636).
Institutional Review Board Statement: Not applicable.
Transparency: The authors state that the manuscript is honest, truthful, and transparent, that no key aspects of the investigation have been omitted, and that any differences from the study as planned have been clarified. This study followed all writing ethics.
Competing Interests: The authors declare that they have no competing interests.
Authors' Contributions: All authors contributed equally to the conception and design of the study. All authors have read and agreed to the published version of the manuscript.
Disclosure of AI Use: The authors used OpenAI's ChatGPT (GPT-5.1) to assist with editing and refining the wording of the manuscript. All content was carefully reviewed and verified by the authors.

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