Development and optimization of an efficient method to detect the authenticity of edible oils

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1. Introduction

Edible oils include the following: soybean oil, maize oil, sunflower oil, peanut oil, sesame oil, rapeseed oil, olive oil and a variety of blended oils, and they can provide essential fatty acids, such as linoleic acid and α-linolenic acid, and the fat-soluble vitamins A, D, E and K [http://en.wikipedia.org/wiki/Vegetable_fats_and_oils]. As technology advances and people’s health awareness increases, people are more concerned about food quality and safety. Thus, food authenticity and traceability have become very important to allow consumers to make informed choices about the foods they buy and eat (Giménez, Pistón, Martín, & Atienza, 2010). Edible oils can be misdescribed with the substitution of one ingredient by a similar but cheaper one or by over-declaring a quantitative ingredient (Woolfe & Primrose, 2004). Thus, the identification of the raw materials in edible oils is important for authentication.

The authenticity of edible oils has been addressed using different techniques such as gas chromatography, infrared spectroscopy, high-performance liquid chromatography, the isotope ratio method (Aluyor, Ozigagu, Oboh, & Aluyor, 2009; Verleyen et al., 2001), proton transfer reaction mass spectrometry (PTR-MS) and nuclear magnetic resonance spectroscopy (NMR) (Giménez et al., 2010). However, these methods are based on the physical and chemical properties of edible oils, and the detection limits of these methods are not sufficient to ensure edible oil authenticity. Thus, there is a growing interest in the application of DNA-based detection methods, such as PCR and real-time PCR, to evaluate edible oil authenticity. Specific protocols for the detection of DNA isolated from soybean oil (Bogani et al., 2009; Costa, Mafra, Amaral, & Oliveira, 2009; Costa, Mafra, Amaral, & Oliveira, 2010; Gryson, Ronse, & Sweettinck, 2004), maize oil (Pauli, Liniger, Zimmermann, & Schrott, 2000), rapeseed oil (Hellebrand, Nagy, & Moërsel, 1998), olive oil (Busconia et al., 2003; Consolandi et al., 2008; Giménez et al., 2010; Spaniolas et al., 2008; Wu et al., 2008) and palm oil (Zhang et al., 2009) have been developed.
Other DNA-based detection techniques used for edible oils include multiple PCR (Giménez et al., 2010), capillary electrophoresis (Ayed, Kamoun, Moreau, & Rebai, 2009; Gíménez et al., 2010) and biosensors (Bogani et al., 2009). A variety of molecular markers (Kamoun, Moreau, & Rebaï, 2009; Giménez et al., 2010) and multiple PCR (Giménez et al., 2010), capillary electrophoresis (Ayed et al., 2007) have been tested for DNA detection. However, these methods are limited in their sensitivity and specificity for the detection of DNA in edible oils.

2. Materials and methods

2.1. Materials

Five different refined edible oils, soybean, maize, sunflower, peanut and sesame oil, were bought from local markers in Beijing.

2.2. DNA extraction

To identify critical aspects of the DNA extraction from the edible oils, DNA isolation from five different edible oils was carried out with two extraction replicates in five sets of parallel experiments to examine the type and the amount of organic reagents and the types and the amounts of extraction buffers and DNA carriers.

2.2.1. The type of organic reagent

We used hexane, heptane and chloroform to determine the most suitable organic reagent for the extraction of DNA from five different edible oils.

DNA from 30 mL of oil was extracted by magnetic stirring for 3 h with 30 mL of organic reagent and then mixing with the CTAB buffer (2% CTAB, 0.8 M NaCl, 50 mM Tris–HCl, 1 mM EDTA) for another 3 h. The sample was centrifuged at 11,000 rpm for 10 min. The aqueous phase was transferred to a 50-mL tube and was then centrifuged at 11,000 rpm for 10 min again, and the aqueous phase was transferred to a new 50-mL tube. The DNA was precipitated by mixing samples with 1 V of absolute isopropanol, 1/10 V of 3 M NaAc (pH 5.2) and 1/1000 V of acryl carrier, followed by incubating at −20°C overnight. The samples were then pipetted into a 2-mL tube and centrifuged at 14,000 rpm for 20 min, and the liquid phase was discarded. The residues in all of the tubes were dissolved in 1 mL of TE. Phenol/chloroform/isoamylol (25:24:1) was added, and the mixture was centrifuged at 12,000 rpm for 10 min. Next, 800 μl of the upper part of the solution was transferred to a new tube, and 1 V of absolute isopropanol and 1/10 V of 3 M NaAc (pH 5.2) were added. The samples were incubated at −20°C for 3 h and centrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was discarded, and the residue was washed with 700 μl of 70% ethanol, centrifuged at 14,000 rpm for 10 min and dried at room temperature. Dried DNA pellets were resuspended in 50 μl of TE.

2.2.2. The amount of organic reagent

The volumes of the organic reagents were 20 mL, 25 mL, 30 mL and 35 mL. The other procedures were the same as the procedures described in Section 2.2.1.

2.2.3. The type of extraction buffer

The extraction buffer used in the extraction method was substituted by the buffers as follows: TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0), 2% CTAB (2% CTAB, 0.8 M NaCl, 50 mM Tris–HCl, 1 mM EDTA), 5% CTAB (5% CTAB, 0.8 M NaCl, 50 mM Tris–HCl, 1 mM EDTA), 1.4 M NaCl (2% CTAB, 1.4 M NaCl, 50 mM Tris–HCl, 1 mM EDTA). The other procedures were the same as the procedures described in Section 2.2.1.

2.2.4. The amount of the extraction buffer

The volumes of the extraction buffer used were 10 mL, 17.5 mL, 25 mL and 32.5 mL. The other procedures were the same as the procedures described in Section 2.2.1.

2.2.5. DNA carriers

In this study, four different DNA carriers, including three commercially available DNA carriers (acryl carrier, glycogen, yeast tRNA) and one DNA carrier that was the DNA extracted from another species in our lab, were used.

We performed 15 parallel experiments examining the DNA carriers described in Table 1, with sunflower oil as the representative edible oil to determine the best DNA carriers for DNA extraction from edible oils.

2.3. Primers

To assess the quality and quantity of the extracted DNA, we utilized primers for amplification of species-specific endogenous genes (Table 2). The primers for real-time PCR were designed using Primer Express software 3.0. All of the primers were synthesized by Sangon Biotech Co., Ltd. (Beijing).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Combinations of DNA carriers.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>S</td>
</tr>
<tr>
<td>G</td>
<td>N</td>
</tr>
<tr>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>D</td>
<td>N</td>
</tr>
</tbody>
</table>

A: Acryl carrier; G: Glycogen; Y: Yeast tRNA; D: DNA extracted from other species. S: DNA carriers included in the combination. N: DNA carriers not included in the combination.
2.4. Qualitative PCR

The PCR reagents for each 30 μl reaction included the following: 2 μl DNA, 1 × PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mM MgCl2), 0.2 mM dNTPs, 0.3 μM primer-F/primer-R and 2.5 units of rTaq DNA Polymerase (Takara Biotechnology Co., Ltd., China). The PCR was carried out under the following program in a thermocycle system gradient (Applied Biosystems, USA): pre-incubation at 95 °C for 5 min and 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; at the end of 35 cycles, the reaction mixtures were incubated for an additional 10 min at 72 °C prior to cooling to 4 °C. The PCR products were detected using 2.5% agarose gels in TAE buffer used for the quantitative SYBR Green assay was as follows: 95 °C for 10 min and 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 68 °C for 30 s, with collection of the fluorescence signal at the end of each cycle. Dissociation curves allow the operator to assess by qualitative PCR, which amplified a species-specific endogenous gene (Fig. 1I). For soybean oil and sunflower oil, expected amplification fragments appeared in all extracts, and the amplified bands of the extracts using chloroform were stronger than the others (Fig. 1I A, C). For maize oil, hexane was obviously the only suitable organic reagent (Fig. 1I B). For peanut oil and sesame oil, the expected amplification fragments were observed in the extracts using heptane and chloroform, and the PCR bands with the DNA extracted using chloroform were stronger than the other bands (Fig. 1I D, E).

We can conclude that the type of organic reagent is a very important factor for extracting DNA from edible oils, especially maize oil. Hexane is required for extracting DNA from maize oil because we could not detect the extracted DNA successfully with heptane and chloroform. On the contrary, hexane, which was most commonly used in previous studies (Bogani et al., 2009), is not suitable for extracting DNA from peanut or sesame oil. Chloroform is the most suitable organic reagent for soybean, sunflower, peanut and sesame oil.

During the DNA extraction process, the mixture of chloroform and oil is in the lower layer when oil and water were separated by centrifugation due to their different densities. In contrast, with hexane and heptane, the pipette does not penetrate the oil phase when drawing up the water phase; consequently, a better effect of oil–water separation was attained using chloroform. In addition, chloroform can remove pigment, phenol and other substances that can inhibit PCR amplification in the water phase.

3. Results

3.1. Optimization of the DNA extraction method for edible oils

3.1.1. The influence of the type of organic reagent on the DNA extraction from edible oils

Great effort has been put forth during the last decade toward the extraction and detection of DNA from edible oils. At present, DNA extraction kits are the main method for extracting DNA from edible oils, and it has been suggested that these kits are effective methods for the extraction of DNA from some edible oils. DNA extraction kits allow for rapid extraction, but they are expensive and not suitable for routine detection. Another DNA extraction method commonly used for extracting DNA from edible oils is the hexane-CTAB method, but this method has been previously shown to lack consistency.

The performance of hexane, heptane and chloroform was assessed by quantitative PCR, which amplified a species-specific endogenous gene (Fig. 1I). For soybean oil and sunflower oil, expected amplification fragments appeared in all extracts, and the amplified bands of the extracts using chloroform were stronger than the others (Fig. 1I A, C). For maize oil, hexane was obviously the only suitable organic reagent (Fig. 1I B). For peanut oil and sesame oil, the expected amplification fragments were observed in the extracts using heptane and chloroform, and the PCR bands with the DNA extracted using chloroform were stronger than the other bands (Fig. 1I D, E).

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3.1.2. The influence of the amount of organic reagents on the DNA extraction from edible oils

In previous studies, the ratio of organic reagent and oil varied greatly, ranging from 1:2 to 6:1 (Bogani et al., 2009; Consolandi et al., 2008; Doveri, Sullivan, & Lee, 2006; Giménez et al., 2010). Fig. 1I shows the PCR amplification results with the extracts in this experiment.

The expected PCR bands appeared in all extracts from sunflower and peanut oil. For sesame and soybean oil, the expected PCR bands appeared in all extracts except those extracted with 35 ml of

| Table 2  
<table>
<thead>
<tr>
<th>PCR primers.</th>
<th>Species</th>
<th>Name</th>
<th>Sequence (5′→3′)</th>
<th>Target</th>
<th>Fragment length (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean</td>
<td>Lec294F</td>
<td>GCCCATCTCGAGGCTTTT</td>
<td>Lectin</td>
<td>294 bp</td>
<td>This study</td>
<td></td>
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<tr>
<td>Soybean</td>
<td>Lec294R</td>
<td>GCCGATCGAAACAAATG</td>
<td>Lectin</td>
<td>233 bp</td>
<td>This study</td>
<td></td>
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<tr>
<td>Soybean</td>
<td>Lec233F</td>
<td>GATGAGACTGATAGAAATGAC</td>
<td>Lectin</td>
<td>118 bp</td>
<td>Yoshimura et al., 2005</td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>Lec233R</td>
<td>CTCTAGCCAGCCCCACATC</td>
<td>Lectin</td>
<td>77 bp</td>
<td>This study</td>
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<tr>
<td>Soybean</td>
<td>Lec114F</td>
<td>GGCCTCTACCTCCCACCGC</td>
<td>Lectin</td>
<td>60 bp</td>
<td>Hernández, Esteve, &amp; Pla, 2005</td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>Lec114R</td>
<td>GGCACCTTCAAAGCTTTTTT</td>
<td>Lectin</td>
<td>60 bp</td>
<td>Hernández, Esteve, &amp; Pla, 2005</td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>Lec77F</td>
<td>TGCCGCTTCCCTCTAACTT</td>
<td>Lectin</td>
<td>60 bp</td>
<td>Hernández, Esteve, &amp; Pla, 2005</td>
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<tr>
<td>Soybean</td>
<td>Lec77R</td>
<td>GCCGATCGAAACAAATG</td>
<td>Lectin</td>
<td>60 bp</td>
<td>Hernández, Esteve, &amp; Pla, 2005</td>
<td></td>
</tr>
<tr>
<td>Sesame</td>
<td>s2sF</td>
<td>GTACCAGAACGGTACGGAT</td>
<td>Lectin</td>
<td>60 bp</td>
<td>Brzezinski, 2007</td>
<td></td>
</tr>
<tr>
<td>Sesame</td>
<td>s2sR</td>
<td>AACTCCGAATGGCCATGAT</td>
<td>Lectin</td>
<td>60 bp</td>
<td>Brzezinski, 2007</td>
<td></td>
</tr>
<tr>
<td>Maize</td>
<td>ZeinF</td>
<td>CGGGTCAATCGAGGCTGAT</td>
<td>Lectin</td>
<td>60 bp</td>
<td>Li et al., 2009</td>
<td></td>
</tr>
<tr>
<td>Maize</td>
<td>ZeinR</td>
<td>AAGGGCCAGTTCTACATCCTT</td>
<td>Lectin</td>
<td>60 bp</td>
<td>Li et al., 2009</td>
<td></td>
</tr>
</tbody>
</table>
organic reagents. Additionally, 25 mL hexane and 25 mL and 30 mL chloroform were optimal for extracting DNA from maize, soybean and sunflower oil (Fig. 1II B, A, C), respectively, because the PCR bands were stronger than the others. No differences were observed between the peanut oil extracts with 30 mL and 35 mL chloroform, but they were stronger than the other two parallel experiments (Fig. 1II D). However, 30 mL chloroform was chosen for peanut oil based on cost saving. The PCR bands of sesame oil extracts with 20 mL, 25 mL and 30 mL chloroform had almost the same intensity, but the bands for 30 mL chloroform were slightly clearer than the other two (Fig. 1II E). Based on the above results, the optimal amount of chloroform for DNA extraction appears to be an equal volume to the volume of edible oil (sunflower, peanut and sesame oils).

3.1.3. The influence of the type of extraction buffer on the DNA extraction from edible oils

The extraction buffers were more diversified than the organic reagents used in previous studies, including two types: one without surfactants (distilled water, TE, TEA, PBS, NaCl solution) and the other one with surfactants (CTAB, SDS, Tween 20, guanidine thiocyanate). The formulations of the extraction buffers utilized in this study were also different than those previously used (Ayed et al., 2009; Busconia et al., 2003; Consolandi et al., 2008; Doveri et al., 2006; Giménez et al., 2010; Gryson et al., 2004; Hellebrand et al., 1998; Torre, Bautista, Cánovas, & Claros, 2004; Wu et al., 2008; Zhang et al., 2009).

Cetyl trimethylammonium bromide (CTAB) was chosen as the representative surfactant because it is commonly used. The mixing of oil and water is actually an emulsification process affected by numerous factors, such as salt and surfactant concentrations. Therefore, we chose different salt and CTAB concentrations to study the influence of the extraction buffer on the DNA extraction from edible oils.

The PCR bands of 5% CTAB were clear in all extracts (Fig. 1III), and the buffer was the optimal extraction buffer for sunflower (Fig. 1III C) and peanut oil (Fig. 1III D). TE was the optimal extraction buffer for sesame oil (Fig. 1III E). Clear PCR bands appeared in all maize oil extracts except for those extracted with 1.4 M NaCl extraction buffer (Fig. 1III B). For soybean oil, the intensity of the

PCR bands for the extracts with 5% CTAB and 1.4 M NaCl extraction buffer was almost the same (Fig. 1II A).

Unlike the organic reagents, the type of extraction buffer was only critical for DNA extraction from sunflower oil.

3.1.4. The influence of the amount of extraction buffer on the DNA extraction from edible oils

Fig. 1IV shows the PCR amplification results for all extracts in this experiment. The optimal amount of extraction buffer for soybean, maize and sesame oil, peanut oil (5% CTAB, 5% CTAB, TE and 5% CTAB) was 25 mL, and the optimal amount for sunflower oil (5% CTAB) was 17.5 mL.

The amount of extraction buffer seriously affects the DNA extraction of four different edible oils. The kind of extraction buffer was most important for maize and peanut oil; without the optimal amount of extraction buffer, the extracts had poor amplification by qualitative PCR. Sunflower oil is almost unaffected by the amount of extraction buffer, followed by soybean and sesame oil.

Too little extraction buffer reduced the efficiency of PCR amplification perhaps because DNA in edible oils was not completely in the water phase or due to the relatively high concentration of PCR inhibitors. When excessive extraction buffer was used, part of the DNA was potentially lost in the DNA extraction process. Therefore, if there is too much PCR inhibition in an edible oil, such as peanut oil, too little extraction buffer may seriously affect the efficiency of the PCR. Excessive extraction buffer is not suitable for almost all kinds of edible oils.

3.1.5. The influence of the DNA carriers in nucleic acid precipitation during the DNA extraction from edible oils

In previous studies, the DNA carrier was added into the aqueous phase (Giménez et al., 2010), but there were some reports that the DNA carrier was added to the oil (Zhang et al., 2009). To determine whether the better extraction step in which to add the DNA carrier and whether DNA carriers played an indispensable role in the DNA extraction process. Therefore, if there is too much PCR inhibition in an edible oil, such as peanut oil, too little extraction buffer may seriously affect the efficiency of the PCR. Excessive extraction buffer is not suitable for almost all kinds of edible oils.

In previous studies, the DNA carrier was added into the aqueous phase (Giménez et al., 2010), but there were some reports that the DNA carrier was added to the oil (Zhang et al., 2009). To determine which was the best DNA carrier for DNA extraction from edible oils, three parallel experiments were performed: one with no DNA carrier, one in which the DNA carrier was added to the water phase and one in which the DNA carrier was added to the oil. PCR amplification was stable when the DNA carriers were stable into the water phase (data not shown).

When a single DNA carrier was used, the clearest PCR bands appeared with yeast tRNA (Fig. 1V M). For the combinations of two different DNA carriers, the extracts with the NO. 9 and NO. 10 combinations were better than the others (Fig. 1V N). For the combinations of three or four different DNA carriers, the intensity of the PCR bands for the NO. 11 combination was the strongest (Fig. 1V K).

Because the intensity of PCR bands is vulnerable to differences in agarose gel electrophoresis, the PCR products of DNA carrier combination 4, 9, 10, and 11 were run on the same agarose gel. The result showed that yeast tRNA was the best DNA carrier for DNA extraction from edible oils (Fig. 1V S).

3.2. Real-time PCR

To estimate the DNA amount and evaluate the DNA extraction method used in this research, the extracts of soybean oil obtained with the optimized method were amplified by SYBR Green real-time PCR.

The DNA from 80 mL, 30 mL and 15 mL soybean oil was amplified by real-time PCR with lec-294-F/lec-294-R, lec-233-F/lec-233-R, lec-118-F/lec-118-R, and lec-77-F/lec-77-R (Table 2) with two PCR replicates. The melting curves of the real-time PCR are presented in Fig. 2. The peaks of the 294 bp and 233 bp amplification fragments of the lectin gene were both observed with 80 mL soybean oil but not with 30 mL soybean oil. The peaks of the 118 bp amplification fragments were present in all extracts. The PCR product amount was reduced with 15 mL soybean oil; the melting curves of the primer dimers were higher than the melting curves of the amplified DNA fragments. A 77 bp amplification fragment also appeared in all extracts. Even with the 15 mL soybean oil, there was only a single peak, which represented the specific PCR product. Thus, the size of the amplified DNA fragments played an important role in the detection of the DNA when obtaining excessively degraded DNA from highly processed matrices. Based on the above conclusion, we chose lec-77-F/lec-77-R for the following experiments.

For quantitative analysis, a standard curve was prepared using an extract of soybean seed serially diluted containing 100–0.16 ng with three PCR replicates. The linear correlation coefficient of the standard curve ($R^2$) was 0.991, and the PCR efficiency ($E$) was 95.737. The real-time PCR amplification results of the oil samples tested in this work are presented in Table 3. The results confirmed the positive detection of the lectin gene in all extracts. Experiments using blank extractions as controls confirmed the absence of DNA contamination.

We also compared the DNA extraction method used in a previous study (Giménez et al., 2010) with the optimized DNA extraction method from our study: the results of the real-time PCR are presented in Table 3. The optimized DNA extraction method developed in this study greatly increased the yield of DNA extracted, especially for a large amount of samples.

With the DNA extraction method of the previous study, the DNA concentration of the extracts from 100 mL soybean oil was lower than that of the extracts from 100 mL blended oil and 80 mL soybean oil. This difference may be because there were many PCR inhibitors in the extracts with 100 mL pure soybean oil. However, the high volume of sample may be needed to detect adulterated ingredients. Therefore, the DNA extraction method in the previous study is not suitable for detecting adulterated edible oils.

The conversion of mass to genome copy number is based on the haploid genome mass (1C value) obtained from the Plant DNA C-values Database for Glycine max and is 1.13 pg (Rott, Lawrence, Wall, & Green, 2004). Therefore, 13,274 DNA copies, 10,088 DNA copies, 4159 DNA copies and 3115 DNA copies were detected from 100 mL, 80 mL and 30 mL soybean oil and 100 mL blended oil, respectively.

Because 5 μL of DNA solution was used as the DNA template in real-time PCR, and the extracted DNA was dissolved in 50 μl TE, there were 1,327, 1,261, 1,396 or 311 DNA copies/mL soybean oil or blended oil, respectively.

Mixtures of soybean oil and sesame oil were used for real-time PCR to simulate the amplification of adulterated edible oils (Table 4) with three PCR replicates. The DNA concentration with the DNA extraction method developed in this study was much greater (~15 times) than that with the DNA extraction method used in the previous study. Because the amount of soybean oil (30 mL) was the same in these three mixtures containing different amounts of sesame oils, the DNA concentration of extracted soybean DNA should be the same, but this was not the case. The DNA concentration decreased with greater amounts of sesame oil. It is possible that some substances in sesame oil, such as pigments, may affect the efficiency of the DNA extraction and the real-time PCR. The relative standard deviation value of the DNA concentration with the DNA extraction method developed in this study was 0.012, which was smaller than that with the DNA extraction method used in the previous study (0.127). We can conclude that the DNA extraction method developed in this study is much more effective and results in a more consistent quantity of DNA than the DNA extraction method used in the previous study.
The soybean DNA concentration from adulterated oil is greater than the DNA concentration from soybean oil with the DNA extraction method developed in this study when extracting the same volume of soybean oil (Tables 3 and 4). The reason for this discrepancy may be that the sample used in Table 4 was only kept at room temperature for 5 days from the product date, but the sample used in Table 3 was kept at room temperature for 7 months.

The DNA in the oil was degraded during storage time; the DNA in oil should be extracted immediately after production. However, the difference in the DNA concentrations with the DNA extraction method used in the previous study was the opposite, indicating that the DNA extraction method used in the previous study is not suitable for mixtures of soybean and sesame oil. PCR inhibitors in sesame oil may not have been successfully removed, resulting in the reduced efficiency of PCR.

We also examined the DNA concentration in adulterated oil with 10 mL soybean oil. The melting curves of real-time PCR are

Table 3
Real-time PCR results for oil extracts.

<table>
<thead>
<tr>
<th>Sample size (mL)</th>
<th>PCR Ct DNA concentration (ng)</th>
<th>PCR Ct DNA concentration (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean oil</td>
<td>29.68 0.30 24.3 15.00</td>
<td>25.04 8.76 24.67 11.40</td>
</tr>
<tr>
<td>15</td>
<td>0.34 4.22 25.89 4.70</td>
<td>0.34 4.22 25.89 4.70</td>
</tr>
<tr>
<td>Blended oil</td>
<td>28.81 0.99 26.29 3.52</td>
<td>28.39 0.86 24.32 14.99</td>
</tr>
<tr>
<td>30</td>
<td>23.3 7.78 22.2 3.76</td>
<td>23.3 7.78 22.2 3.76</td>
</tr>
</tbody>
</table>

* Multiple Tm peaks.

Table 4
Real-time PCR results for adulterated oil extracts.

<table>
<thead>
<tr>
<th>Soybean oil (mL)</th>
<th>Sesame oil (mL)</th>
<th>DNA extraction method used in the previous study (CTAB)</th>
<th>Optimized DNA extraction method developed in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>40</td>
<td>28.04 0.98</td>
<td>24.28 15.16</td>
</tr>
<tr>
<td>30</td>
<td>70</td>
<td>28.39 0.86</td>
<td>24.3 14.99</td>
</tr>
<tr>
<td>30</td>
<td>100</td>
<td>28.22 0.76</td>
<td>24.32 14.80</td>
</tr>
</tbody>
</table>
presented in Fig. 3. Bimodal melting curves were observed in all extracts, even though there was only the primer dimer peak in the mixture of 10 mL soybean oil and 40 mL sesame oil. The PCR products were obviously reduced, especially for the DNA from 40 mL sesame oil. The volume of the oil sample should be large enough to reach the detection limit of PCR.

3.3. Gold colloid real-time PCR

Gold colloid is a novel material with many physical properties, and it can enhance the efficiency and the sensitivity of PCR. To determine the effective concentration of gold colloid, real-time PCR with different amounts of gold colloid was performed with serially diluted soybean DNA (20 ng, 4 ng, 0.8 ng, 0.16 ng and 0.032 ng) with seven PCR replicates. As shown in Fig. 4, 0.08 μl of gold colloid did not make an obvious difference in the efficiency of real-time PCR, but all of the other concentrations of gold colloid made an obvious difference in the efficiency of real-time PCR, especially for low concentrations of DNA. With gold colloid, the efficiency of the PCR was increased by 0.4–1.06 cycles for 0.16 ng and 0.032 ng DNA and by 0.08–0.71 cycles for 0.8 ng, 4 ng and 20 ng DNA. In 7 sets of parallel experiments, the efficiency of the PCR with 0.16 ng and 0.032 ng of DNA was increased, but this did not always occur for 0.8 ng, 4 ng and 20 ng DNA; sometimes the PCR was inhibited by the gold colloid (data not shown). The efficiency of real-time PCR with low amounts of DNA can be increased by gold colloid. The efficiencies of the standard curves of real-time PCR with gold colloid were all increased, mainly due to the smaller Ct values of low amounts of DNA compared with the PCR without gold colloid (Fig. 4). The R² values of the standard curves of real-time PCR with 0 μl, 0.2/6 μl, 0.3/6 μl and 0.4/6 μl gold colloid were all over 0.99, except for 0.1/6 μl and 0.5/6 μl. The effective concentration of gold colloid was experimentally determined to be 0.2/6–0.4/6 μl.

The DNA of the adulterated edible oils, which were the mixtures of 10 mL soybean oil and 40 mL sesame oil, was amplified by real-time PCR with 0 μl, 0.2/6 μl, 0.3/6 μl and 0.4/6 μl gold colloid (Fig. 5) with three PCR replicates. The efficiency of the real-time PCR with 0.2/6 μl, 0.3/6 μl and 0.4/6 μl gold colloid was dramatically increased by 0.66–2.76 cycles. The standard deviations of the Ct values of real-time PCR with and without gold colloid were 0.052–0.292 and 0.628–1.186, respectively. The DNA of the edible oils was more precisely detected by real-time PCR with colloid than without colloid.

The Ct values of real-time PCR that amplified the edible oils, except for 10 mL soybean oil DNA kept at 4 °C overnight, were 29.44–32.9 and were similar to the Ct values of real-time PCR that amplified 0.032 ng and 0.16 ng DNA of soybean seed, which were 30.25–32.79. However, the efficiency of real-time PCR that amplified the DNA of edible oils was increased by 0.66–2.76 Ct values, more than the Ct values of the DNA of soybean seed, which were 0.4–1.06 cycles. Thus, the efficiency of real-time PCR with poor quality DNA was increased more than that with good quality DNA.

4. Discussion

With the emergence of various issues regarding edible oils, analytical methods are needed for the identification of products for consumer protection as well as product safety.

In a previous study, the detection methods based on the DNA from edible oils mostly focused on one kind of edible oil. In this study, the optimal DNA extraction methods for five different edible oils were determined. The method used organic reagents chosen due to their successful application with several refined vegetables oils. There is not a suitable DNA extraction method for all kinds of edible oils. However, we obtained a DNA extraction method for soybean, sunflower, peanut and sesame oil but not maize oil. In this method, chloroform is used as the organic reagent, the amount of the organic reagent is equal to the volume of the edible oil, the extraction buffer is 5% CTAB and the volume ratio of the extraction buffer to edible oil is 5:6. The types of organic reagents and DNA carriers determine whether DNA can be successfully extracted from different edible oils. Although hexane was also a suitable organic reagent for DNA extraction from soybean oil, too much foam was produced due to the high speed of magnetic stirring when the sample size was 100 mL, causing a decrease in the DNA concentration. Additionally, phenol/chloroform/isoamyl alcohol (25:24:1) is an essential step for DNA extraction from refined oil and is better than phenol/chloroform (24:1) (data not shown). The step was even performed during the extraction using a kit by Pafundo, Busconi, Agrimonti, Fogher, and Marmiroli (2010). When the sample size is large, more care should be taken in the extraction process, especially when dissolving the residue with TE. After centrifugal sedimentation, the DNA precipitation should not be washed too many times, otherwise too many PCR inhibitors will be dissolved simultaneously, and the efficiency of PCR will be influenced.

The DNA extraction method developed in this study greatly increased the efficiency of the extraction process compared with the result of the research of Costa et al., (2008) which is the first report on soybean DNA detection from fully refined vegetable oils. We can extract 1396 DNA copies and 311 DNA copies/mL of pure soybean oil or blended oil, far greater than the results of Costa et al.,
who extracted 49.3/53.9/35.0 copies/200 mL blended oil and did not quantify DNA from 200 mL soybean oil. This difference may be because the pre-concentration step is not suitable for refined oils. Additionally, the cost of the method in this study is much lower than the method used by Costa et al. Other issues are sample size and the PCR fragments amplified. Several authors have reported the use of large amounts of oil samples when extracting DNA from edible oils (Costa et al., 2009; Gryson et al., 2004; Hellebrand et al., 1998; Pasqualone et al., 2007). Similarly, for higher percentages of adulteration, successful detection can be obtained with a larger sample size. We were able to detect 118 bp PCR fragments that could not be positively detected by Costa et al. Spaniolas et al. (2010) described a methodology for the identification of the botanical origin with the mixtures of olive oils and sesame oil in a 1:1 (v/v) ratio as sample, using a capillary electrophoresis system. We successfully detected the adulterated oils with higher percentages of adulteration (1:4) with more simple and more common detection technology.

NanoPCR was used to amplify the DNA of edible oils, which was seriously degraded during the production process. The results of this research prove again that nanoPCR can increase the efficiency of PCR amplification when the concentration of gold colloid is in an effective concentration range. The difference in Ct values of the lectin gene with and without gold colloid was greater than 0.5 cycles of the PCR that amplified the BNIP3 gene in colorectal tissue in the research of Li, Lin, Wu, and Liu (2005) but was lower than the PCR that amplified the lectin gene in edible oils. The quality and purity of the DNA may play an important role in nanoPCR.

In conclusion, it is possible to extract trace amounts of amplifiable DNA from refined vegetable oils, such as soybean, maize, sunflower, peanut and sesame oil, and adulterated oils. This new DNA extraction method is a notable achievement due to the lack of published reports demonstrating an efficient DNA extraction method from different refined oils and adulterated oils. The method we developed could aid in the detection of adulterations in refined oils. However, further experiments are necessary to determine the precise quantities of adulterated ingredients and rapid DNA extraction from refined oils.

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