Arabian Journal of Chemistry (2018) **11**, 815–826

King Saud University

Arabian Journal of Chemistry

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New method for effective identification of adulterated *Camellia* oil basing on *Camellia oleifera*-specific DNA

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Received 3 December 2017; accepted 21 December 2017
Available online 28 December 2017

KEYWORDS

Camellia oil;
Specific DNA;
Adulteration identification;
Camellia oleifera;
Fatty acids

Abstract *Camellia* oil obtained from *Camellia oleifera* seeds is rich in unsaturated fatty acids and unique flavors, and has become a rising high-quality edible vegetable oil in the world. However, honored as the "Oriental olive oil", *Camellia* oil was widely adulterated for the situation of high price and short supply. At present, the identification of adulterated plant edible oil is mainly based on the composition and content of fatty acids. Here, the fatty acid composition and content of the main vegetable edible oils were determined. It is found that the fatty acid composition and content are susceptible to the change of the origin, variety and climate of the raw materials, and adulterated oils could even be made extremely similar to *Camellia* oil by the target combination of fatty acid content, therefore it is difficult to accurately identify the adulteration of *Camellia* oil through the composition and content determination of fatty acids. *Camellia oleifera* DNA was used as the breakthrough point for adulteration identification. Basing on the EST library and transcriptome data of *Camellia oleifera*, 116 candidate specific DNAs were screened out by bioinformatics, then the optimized methods of trace DNA extraction in *Camellia* oil were established. Further, three specific *Camellia oleifera* DNAs that could only be PCR amplified using C*amellia* oil- extracted

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.arabjc.2017.12.025

DNA as template were finally screened out, which were confirmed by exclusive PCR amplifications using DNAs of other edible oils as templates. One of the specific DNAs was used to make the concentration regression curves of trace DNA by qPCR (Quantitative real-time PCR). The computational model was successively established between the adulteration ratio and the Ct value of the qPCR by adulteration imitation of different proportions of *Camellia* oil. Finally, a complete identification system of *Camellia* oil adulteration was firstly established basing on the specific DNA of *Camellia oleifera*, and it may provide a new idea and method for identification of adulterated *Camellia* oil.

1. Introduction

Camellia oil is the vegetable oil obtained from *Camellia oleifera* seeds, and is a kind of edible tree-bearing oil with high quality originated from China. *C. oleifera* has been widely cultivated in more than 10 provinces in South China. Some Asian countries such as Vietnam have also started cultivating *C. oleifera* widely in recent years. The content of unsaturated fatty acids in *Camellia* oil is over 85% fromdata prepared by Yang et al. (2016) and Ma et al. (2011), Shamsudin et al. (2017), Rahman et al. (2017), Khan et al. (2017). *Camellia* oil is one of edible high-quality oils recommended by FAO. It's known as oriental olive oil because its fatty acid composition is extremely similar to olive oil. *Camellia* oil also contains polyphenols, sasanquasaponin and other bioactive substances from data prepared by Ye et al. (2014), Sukor et al. (2017), Basheer et al. (2017), Razali and Said, (2017) . Long-term intake of *Camellia* oil can help to cure cardiovascular and cerebrovascular diseases from data prepared by Bumrungpert et al. (2016), reduce the level of cholesterol, and protect the liver from data prepared by Cheng et al. (2015), Ghafar et al. (2017), etc. In addition, through deep processing technology, *Camellia* oil can also be used as a base oil for advanced natural skin care cosmetics from data prepared by Liu et al. (2012), Hassan et al. (2017), Ismail and Hanafiah, (2017).

In recent years, with the improvement of people's living standard, more and more people pursue healthy diet. The increasing demand of *Camellia* oil results in rising price. At the same time, the adulteration of *Camellia* oil becomes more and more serious in China. Over the years, scholarsalways pursued the methods of detecting and identifying edible oil adulteration, such as chromatography was used by Ma et al. (2016), Zhang et al. (2014), Christopoulou et al. (2004), Zhang et al. (2016); Xu et al. (2014) used nuclear magnetic resonance spectroscopy; spectroscopy was used by Farley et al. (2017), Tan et al. (2017), Biswas et al. (2016), Souza et al. (2015), Yuan et al. (2013); Wu et al. (2004) used atomic mass spectrometry and Vietina et al. (2013) used dissolved conversion curve . All the above methods are based on the chemical constituents of edible vegetable oils, such as fatty acids and flavor compounds. However, the content and composition of these chemical constituents will change accompanied with the origin, variety and growth condition of the raw materials. Therefore, it's very difficult to form the unique fatty acid characteristics of *Camellia* oil, resulting in inability to accurately identify whether it is adulterated and what percentage it is.

With the development of gene and genomics technology, the method basing on specific DNA is adopted to detect and identify the adulteration of edible vegetable oil according to Scollo, et al. (2016), Halim and Phang, (2017), Halim et al. (2017), Aziz and Hanafiah, (2017). Different oil plants contain different specific DNA with high genetic stability. This method can effectively avoid the fault of composition change of fatty acids and other components in edible vegetable oil due to changes of external factors in traditional detection methods.

However, there are some difficulties in the adulteration identification of *Camellia* oil basing on the specific DNA. Although there is a method of extracting DNA from olive oil according to Raieta et al. (2015), the preparation process of olive oil and *Camellia* oil is obviously different. Olive oil is extracted by pressing berries, with fewer steps and lower processing temperatures, which will result in less DNA damage during oil production. *Camellia* oil is a fat-soluble substance extracted from seeds. After the complex refining such decolorization, deodorization at high temperature, the content of *C. oleifera* DNA in *Camellia* oil is very low and easy to degrade. Therefore, it is more difficult to extract DNA from *Camellia* oil. Moreover, the genetic background of *C. oleifera* is complex and there is few genome data, which limits the search for specific DNA. Therefore, an effective method of extracting DNA from *Camellia* oil and an effective screening of *C. oleifera* specific DNAs are the basis of identifying adulterated *Camellia* oil basing on specific DNA. In this paper, according to EST Library and transcriptome of *C. oleifera*, the specific *C. oleifera* DNAs that could only be amplified using C*amellia* oil- extracted DNA as template were finally screened out, which were confirmed by exclusive PCR amplifications using DNAs of other edible oils as templates. Finally, a complete identification system of *Camellia* oil adulteration was established basing on *C. oleifera*-specific DNA by qPCR (Quantitative real-time PCR).

2. Materials and methods

2.1. Materials and pretreatment

Vegetable oil samples were provided by Hunan institute of food quality supervision and research, China, then kept at 4 °C (Table 1).

Plant tissue samples: oil palm fruits, rape seeds, the seedlings of *Olea europaea*, soybeans, peanuts, maize, sunflower seeds and leaves of *C. oleifera*.

Leaves of *C. oleifera* were provided by *C. oleifera* resources garden in Central South University of Forestry and Technology and Hunan institute of food quality supervision and research, China. The other materials were purchased in the

Table 1 Samples of edible vegetable oils.

Vegetable oil	Number	Brand	Level	Origin
Camellia oil	DSX01	Dasanxiang	Pressing level I	China
	XTY02	Xintaiyu	Pressing level I	China
	F313	Oil in bulk		China
	GC3171	Oil in bulk		China
	WJ0079	Oil in bulk		China
	ZZ003	Oil in bulk	Crude oil	China
	2016061301	Jinhong	Pressing	China
	2016061302	Jinhong	Pressing	China
	2016061306	Taiyu	Pressing level I	China
	2016061317	Jiajia	Pressing	China
	20160305	Shengyuan	Pressing level II	China
Palm oil	2016061327	Oil in bulk		China
Rapeseed oil	20160301	Yingcheng	Pressing level I	China
	20160302	Guitaitai	Pressing	China
	20160410	Daodaoquan	Pressing level III	China
	20160411	Yingcheng	Pressing level I	China
	20160415	Oil in bulk		China
	2016061308	Shengyuan	Pressing	China
	2016061319	Xiyouduo	Pressing	China
Olive oil	20160017	AGRIC	Extra virgin	Greece
	20160015	Olivoila	Blend	China
	20160021	BELLINA	Extra virgin	Spain
Soybean oil	20160402	Jinlongyu	Leaching grade I	China
	2016061322	Meishishang	Leaching grade I	China
	20160306	Hongsui	Pressing	China
	20160403	Wuhu	Leaching grade I	China
Peanut oil	20160412	Luhua	Pressing level I	China
	20160303	Huoniao	Pressing level I	China
	20160413	Jinlongyu	Pressing level I	China
	20160416	Oil in bulk		China
Maize oil	20160408	Changshouhua	Pressing level I	China
	20160409	Runzhijia	Pressing level I	China
	2016061304	Panzhongcan	Pressing level I	China
	2016061314	Xiwang	Pressing	China
Sunflower seed oil	20160405	Daodaoquan	Pressing level I	China
	20160304	Beitaiyupin	Pressing	China
	20160406	Duoli	Pressing level I	China
	20160407	Runzhijia	Leaching grade I	China

market. The plant tissue samples were washed in deionized water and stored in the ultra-low temperature refrigerator.

2.2. Methods

2.2.1. Bioinformatics analysis of characteristics of C. Oleifera DNA

A large number of new genes (more than 2000) were revealed by the *C. oleifera* EST library and transcriptome obtained from previous studies. These new genes were not homologous with the existing DNAs in GenBank.

NCBI CDD was used to analyze the conserved domains in the amino acid sequence, ClustalX was used for amino acid multi-sequence alignment, and MAGE5 was used to construct phylogenetic trees. Through the systematic bioinformatics analysis of the new genes, only single-copy genes without obvious hairpin structure were selected as candidates of *C. oleifera* specific DNAs.

2.2.2. Design of specific primers for C. oleifera-specific DNA

According to the DNA sequence characteristics of the new C. oleifera gene selected via bioinformatics analysis, the specific primers were designed according to the conserved DNA sequence. In order to avoid multiple sequences amplification caused by introns, the forward and reverse primers of these new genes were designed in the same exon. Primers were designed by Primer Premier 5.0. The target lengths of amplified fragment were about 200–500 bp.

2.2.3. DNA extraction of Camellia oil

Camellia oil is a fat-soluble sample with a low content of DNA. It is quite difficult to obtain enough DNA by a conventional DNA extraction. Thus, DNA in *Camellia* oil was extracted using the modified SDS, CTAB and 2× CTAB methods, and the concentration and purity of extracted DNA were determined by nucleic acid protein analyzer (BIO-RAD SmartSpec Plus Spectrophotometer). The extracting efficiency of

three methods was compared to select a suitable method of extracting DNA from *Camellia* oil.

2.2.3.1. Enriching DNA in Camellia oil. The content of DNA in *Camellia* oil is extremely low, and it is very difficult to extract DNA directly, so DNA enrichment must be carried out firstly. After several optimization, the enriching method of DNA in *Camellia* oil was established as followed: 1 ml *Camellia* oil and 400 µl TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)) were added in a 2 ml tube, centrifuged at 13,000 rpm for 3 min, and the supernatant oil was removed, then 1 ml *Camellia* oil was again added into the same tube. The above steps were repeated 20 times to enrich DNA of 20 ml *Camellia* oil into 300–400 µl aqueous phase. Each *Camellia* oil was enriched simultaneously in two tubes, then mixed to form the final DNA-enriched sample.

2.2.3.2. DNA extracting methods for DNA-enriched sample.

(a) Modified SDS method: 100 µl 20% SDS was added to the DNA-enriched sample of *Camellia* oil and mixed thoroughly. The mixture was heated at 65 °C for 30 min and mixed by inverting every 5 min. Then 200 µl 3 M NaAc was added and mixed, centrifuged at 15,000 rpm, 4 °C for 10 min. The supernatant was taken and added the same volume of chloroform/isoamyl alcohol (24: 1) to remove proteins once (it could be repeated again if obvious impurities existed.); Add isopropyl alcohol to precipitate DNA at -20 °C for 1 h. The precipitation was washed using 70% ethanol once or twice, after dried, then dissolved in 50 µl TE.

(b) Modified CTAB method: 500 µl CTAB extraction buffer (2% CTAB, 0.1 M Tris-HCl (pH 8.0), 20 mM EDTA(pH 8.0), 1.4 M NaCl, 0.2% β-Mercaptoethanol) was added to the tube with the DNA-enriched sample, after mixed, kept in 65 °C water bath for 30–60 min, gently shaken every 10 min. Add the same volume of chloroform/isoamyl alcohol (24:1), mix by inverting, then centrifuge at 12,000 rpm for 10 min, which will be repeated once or twice depending on the impurity volume. Take the supernatant, add the double volume of anhydrous ethanol, then centrifuge at 12,000 rpm for 10 min after precipitated at −20 °C for 1 h. Add 350 µl TE to resuspend the precipitate, add 1/10 volume of 3 mol/L NaAc and the double volume of pre-cooled ethanol to precipitate DNA at −20 °C for 1 h. Remove the supernatant after centrifugation at 12,000 rpm for 10 min, and then

Table 2 Fatty acids composition of common vegetable oil (%).

Vegetable oil	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid	Arachidonic acid	Behenic acid
Palm oil	45.137	4.395	39.734	8.742	0.148	0.338	0.056
Rapeseed oil	4.995 ± 1.534	1.964 ± 0.192	65.283 ± 8.539	19.531 ± 6.506	5.748 ± 3.405	0.490 ± 0.099	0.282 ± 0.035
Olive oil	11.713 ± 1.075	2.165 ± 0.486	76.129 ± 3.664	6.950 ± 2.829	0.636 ± 0.061	0.367 ± 0.020	0.101 ± 0.008
Soybean oil	10.673 ± 0.323	3.799 ± 0.389	23.471 ± 0.793	54.580 ± 0.973	5.873 ± 0.686	0.302 ± 0.013	0.346 ± 0.006
Peanut oil	10.711 ± 0.671	3.261 ± 0.501	43.526 ± 3.072	36.749 ± 2.590	0.904 ± 0.554	1.257 ± 0.164	2.120 ± 0.153
Maize oil	12.202 ± 0.654	1.590 ± 0.118	30.332 ± 2.379	54.032 ± 3.203	0.849 ± 0.681	0.332 ± 0.044	0.104 ± 0.026
Sunflower Seed oil	6.134 ± 0.330	2.915 ± 0.118	30.220 ± 7.508	57.545 ± 10.704	1.720 ± 2.905	0.222 ± 0.084	0.503 ± 0.100
Camellia oil	8.432 ± 0.393	1.895 ± 0.134	80.150 ± 0.316	8.391 ± 0.391	0.272 ± 0.195	ND	ND

Note: ND represents that content is less than 0.05%.

Table 3 Effect of extracting DNA from Camellia oil.

Methods	Samples	Average value OD_{260}	Average value OD_{280}	Purity of DNA	Concentration of DNA (ng/µl)
Modified SDS	F313	0.014	0.009	1.56	0.70
	GC3171	0.009	0.006	1.50	0.45
	QW1546	0.010	0.006	1.67	0.50
Modified CTAB	F313	0.005	0.005	1	0.25
	GC3171	0.004	0.004	1	0.20
	QW1546	0.014	0.014	1	0.70
Secondary CTAB	F313	0.021	0.018	1.17	1.05
	GC3171	0.027	0.025	1.08	1.35
	QW1546	0.013	0.011	1.18	0.65

the precipitate was washed with 200 µl 70% ethanol twice. After dried in ventilation cabinet at room temperature for about 40 min, the precipitate was dissolved in 50 µl TE.

(c) Secondary CTAB method: 750 µl 65 °C-preheated 10 × CTAB buffer (10% CTAB, 75 mM Tris-HCl (pH 8.0), 15 mM EDTA (pH 8.0), 1 M NaCl, 1% β-Mercaptoethanol) was added to 600 µl DNA-enriched sample, then incubated at 65 °C for 30–60 min with inverting mixing every 10 min. Add 750 µl chloroform/isoamyl alcohol (24:1), then centrifuge at 14,000 g for 10 min after mixing by inverting for 5 min. Repeat this step once. Add 1.5 volumes of CTAB sedimentation buffer (1% CTAB, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH8.0)) to the supernatant, precipitate for 10 min after mixing, then centrifuge at 14,000 g for 20 min. After removing supernatant, the precipitate was dried in room temperature for 15–30 min, which followed by washing with 70% ethanol. The precipitate was dissolved in 50 µl TE finally.

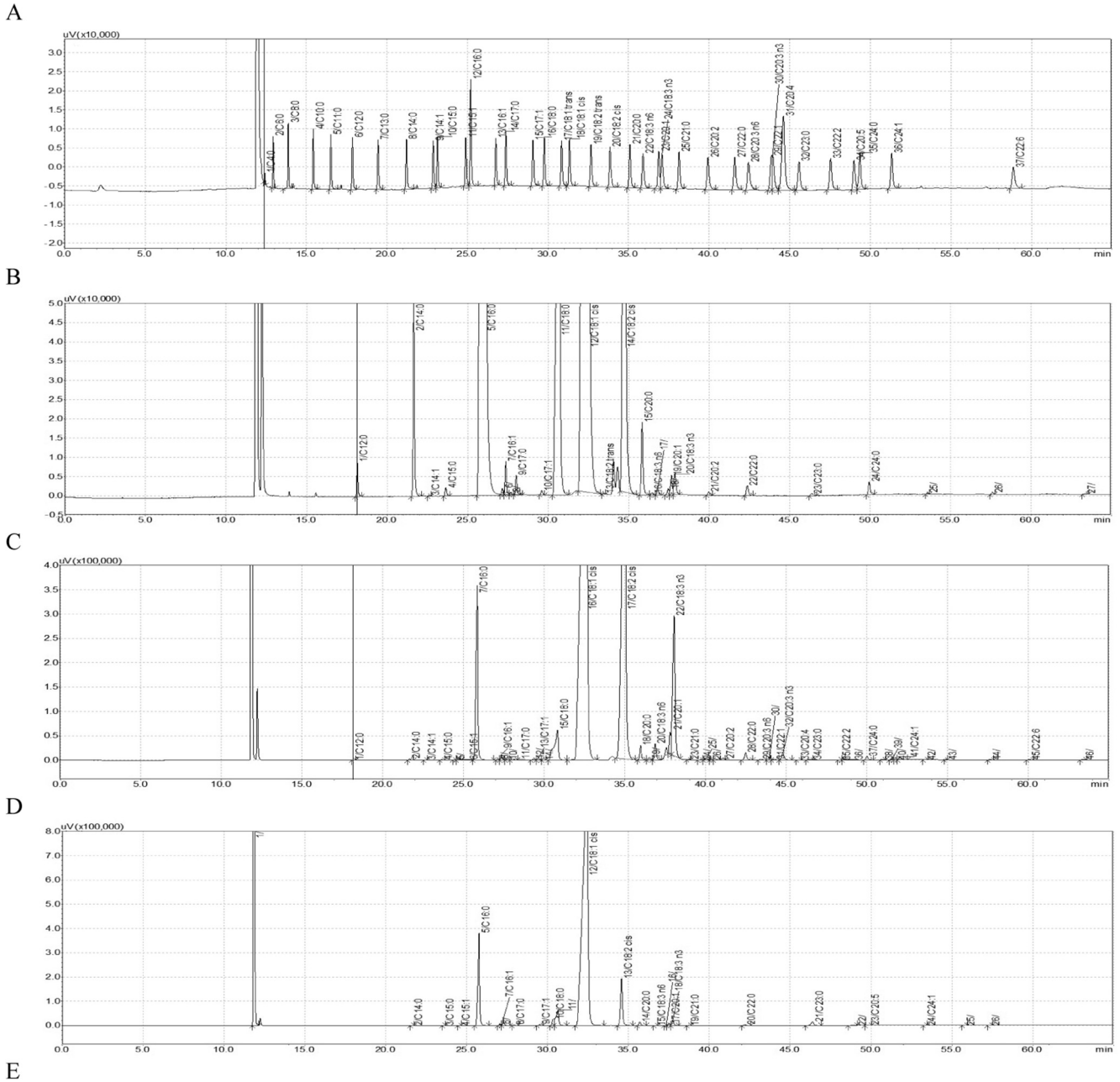

Fig. 1 GC diagram of fatty acid composition of eight edible vegetable oils (A: standard, B: palm oil, C: rapeseed oil, D: olive oil, E: soybean oil, F: peanut oil, G: maize oil, H: sunflower seed oil, I: *Camellia* oil).

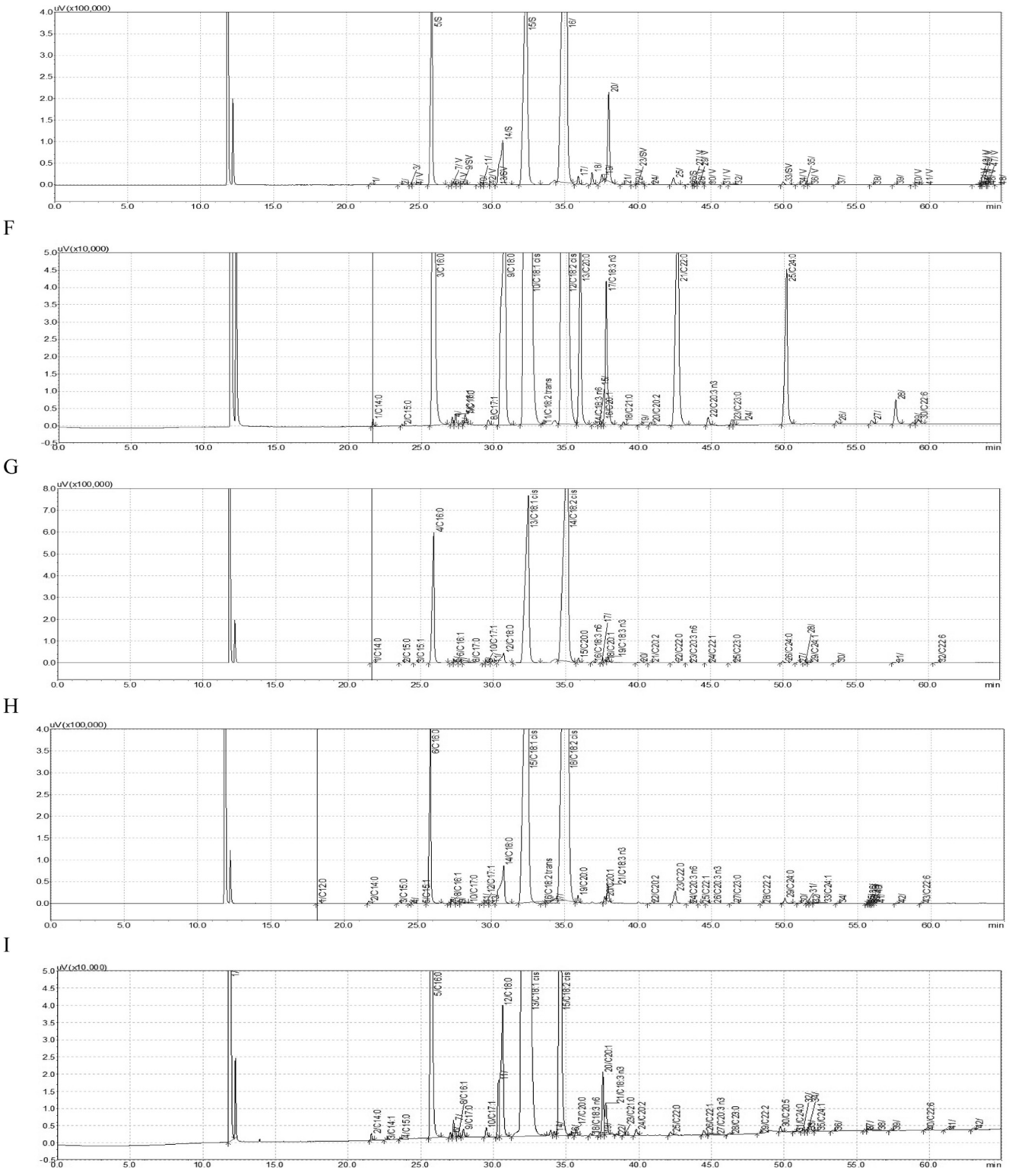

Fig. 1 (*continued*)

2.2.4. Primers screening for specific PCR amplification

Basing on rules of stable and specific amplification, the candidate primers were screened by PCR using enriched DNAs of five *Camellia* oil as templates. Because the DNA content in *Camellia* oil is low, an optimized PCR system was developed.

PCR system (10 μl): 5 μl 2×EasyTaq PCR SuperMix (+dye) (purchased from Beijing Transgen Biotechnology Co., Ltd.), 0.5 μl positive primers (10 μM), 0.5 μl reverse primers (10 μM), 1 μl template DNA, 3 μl ultrapure water.

PCR program (touch-down PCR): pre–degeneration (94 °C/3 min), followed by 35 cycles: denaturation (94 °C/30 s), annealing (initial 65 °C/30 s, temperature reduced by 2 °C per 5 cycles), extension (72 °C/45 s). Finally keep at 72 °C for 10 min.

The above PCR program was completed by the BIO-RAD S1000™ Thermal Cycler. Gel imaging system was BIO-RAD Gel Doc™ XR + Gel Documentation System.

2.2.5. Exclusive screening of candidate Camellia oil DNA-specific primers

In order to ensure that these screened specific primers can only be PCR amplified specifically in *Camellia* oil DNA, it is necessary to perform exclusive PCR screening of these screened primers using DNAs from edible vegetable oils to avoid false positive amplification due to the possible gene similarity. First, basing on unique DNA sequence of other seven oil plants (rape, oil palm, olive, sunflower seed, peanut, soybean, maize), seven pairs of primers were designed correspondingly, which were used to verify if the DNAs were successfully extracted from these seven vegetable oils. This will avoid false negativity. The candidate *Camellia* oil DNA-specific primers were used for exclusive amplification, and only primers with no amplified bands in DNAs of other seven oil plants were screened out finally.

The PCR system refers to the PCR system in section ''Primers screening for specific PCR amplification''. PCR program (touch-down PCR): pre–degeneration (94 °C/3 mi n), followed by 35 cycles: denaturation (94 °C/30 s), annealing (initial 66 °C/30 s, temperature reduced by 2 °C per 5 cycles), extension (72 °C/45 s), finally keep at 72 °C for 10 min.

2.2.6. Establishment of qPCR system and regression curve

Basing on the DNA sequence of PCR products by *Camellia* oil DNA-specific primers, the corresponding qPCR primers and probes (TaqMan method) were designed, which synthesized by Nanjing Genscript Biotechnology Co. Ltd. A stable and reliable qPCR system was established by optimizing the reaction system and cycling condition using gradient-diluted *Camellia* oil DNA as templates. On the basis of this, the regression curves of the qPCR results were established using *Camellia* oil DNA with certain gradient concentrations (100%, 10%, 1%, 0.1%, and 0.01%).

qPCR system (25 μl): 12.5 μl Premix Ex Taq (Probe qPCR) (purchased from Takara Biomedical Technology (Beijing) Co., Ltd.), 1 μl primers (10 μM) (positive and reverse primer were 0.5 μl, respectively), 1 μl probe (10 mM), 1 μl template DNA, 8.5 μl ultra-pure water.

qPCR program: pre–degeneration (95 °C/30 s), followed by 60 cycles: denaturation (95 °C/5 s), annealing (60 °C/30 s). Fluorescence detection is performed after each end of the annealing process.

The above qPCR program was performed by the BIO-RAD CFX96 Real-Time PCR Detection System.

2.2.7. Identification of simulated Camellia oil adulteration basing on specific DNA

In order to simulate the adulteration of *Camellia* oil, the DNA of crude oil from *Camellia* seeds was diluted according to the gradients of 20%, 40%, 60%, 80% and 100%. By above qPCR system and program, the gradient samples were amplified to make qPCR curve.

The qPCR system is same with the section ''Establishment of qPCR system and regression curve''. The qPCR program is almost same with the section ''Establishment of qPCR system and regression curve'' except that the number of cycles was reduced to 45.

2.2.8. Fatty acid composition of edible oils by GC

The fatty acid composition of *Camellia* oil and other seven vegetable oils (palm oil, rapeseed oil, olive oil, soybean oil, peanut oil, maize oil, and sunflower seed oil) were determined by GC analysis. 0.5–1 g sample of each oil was add dissolved in 2 ml n-hexane, then was methyl-esterified by adding 2 mol/L potassium hydroxide methanol for 30 min. After centrifugation at 4500 r/min for 5 min, transfer the upper ester solution to a grinding glass bottle. The standards are 37 fatty acid methyl esters from NU-CHEK GLC37 (NU-CHEK PREP, Inc. USA).

GC methods: gas chromatograph was GC-2010 (SHIMADZU, Japan) and the detector was FID. The chromatographic column was CD-2560 (100 m × 0.25 mm × 0.20 μm). The temperature of inlet and detector were 250 °C. The column temperature started at 40 °C for 5 min, heated up to 175 °C at 10 °C/min, held at 175 °C for 10 min, heated up to 210 °C at 5 °C/min, kept for 20 min, heated up to 230 °C at 5 °C/min, and held for 15.5 min. Split ratio was 50:1. Carrier gas velocity: hydrogen: 40 ml/min, tail blowing: 30 ml/min, air: 400 ml/min.

Table 4 Sequence of specific primers for *C. oleifera* DNA.

Primer name	Sequence
124	124F (TCCTAGCACTAGCCCTATTTG), 124R (CTCCTCTTCCCTCATCTCTC)
142	142F (AATCCTACCACTAGCCCTCT), 142R (CTTCTCCTCTTCCCTCATCT)
413	413F (TAGCCCTCTTTGCCCTGATC), 413R (CATTTTCTCCAACTTGTCGC)
420	420F (GCACTAGCCCTCTTTGCCC), 420R (TCCTCTTCCCTCATCTCTCCC)
435	435F (TCAGCCAAACCTCCGCCTT), 435R (TCTCCCCTCTTCTCTCCCTCCT)
494	494F (TCAGCCAAACCTCCGCCTT), 494R (TCCCCTCTTCTCTCCCTCCTG)
908	908F (AATCCTAGCACTAGCCCTCT), 908R (CTCCTGTTCCCTCATCTCTC)
948	948F (AATCCTAGCACTAGCCCTCT), 948R (CTCCTCTTCCCTCATCTCTC)
954	954F (TCCTAGCACTAGCCCTCTTTG), 954R (GGCAGTCCTGGTCCATTTTC)
989	989F (GTACTAGCCCTCTTTGCCCT), 989R (CCTCTTCCCTCATCTCTCCC)
1201	1201F (TCCTAGCACTAGCCCTCTTTG), 1201R (GTCCTGGTCCATTTTCTCCA)
1214	1214F (AATCCTAGCACTAGCCCTCT), 1214R (CTCCTCTTCCCTCATCTCTC)

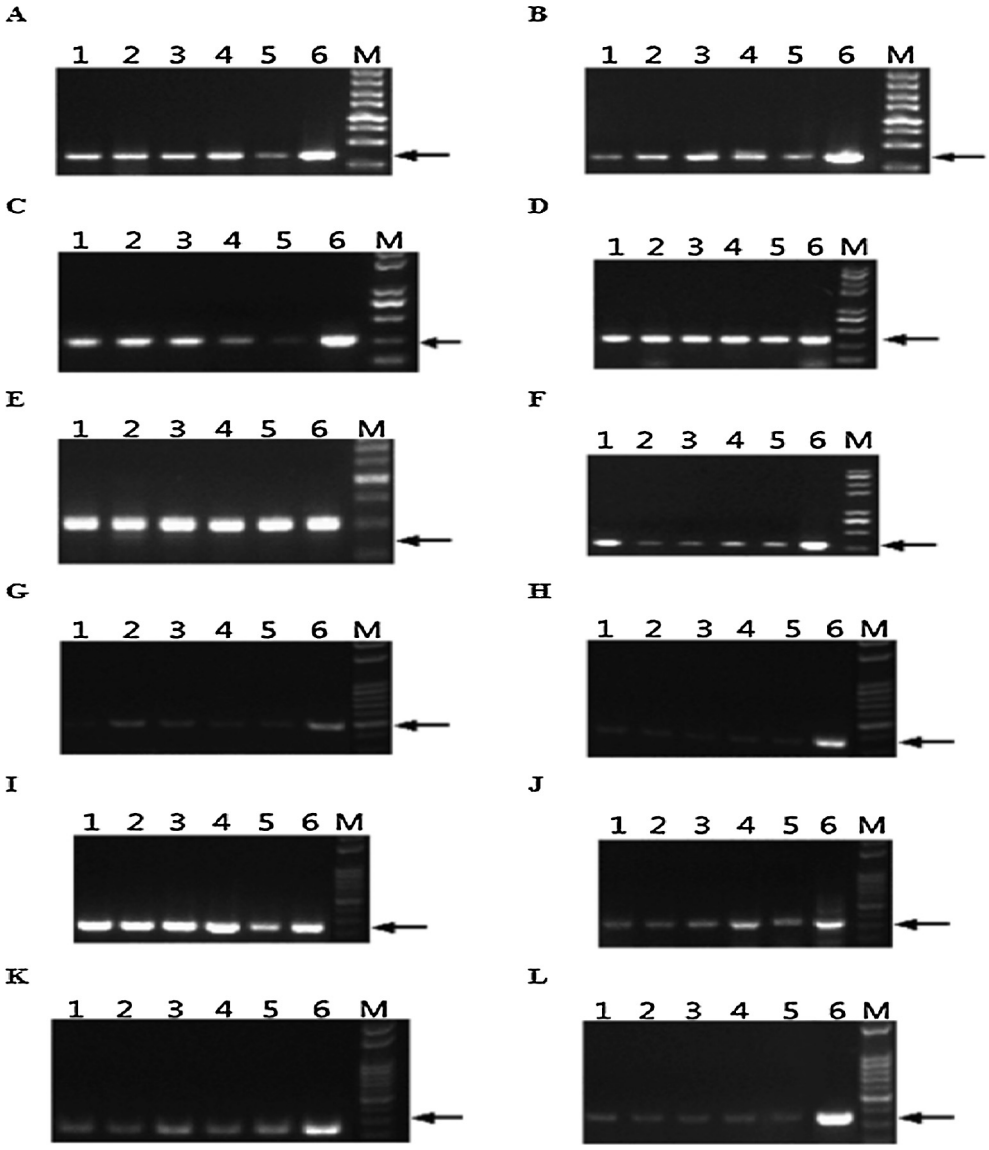

Fig. 2 PCR results by 12 pairs of specific primers (A: primer 124, B: primer 142, C: primer 413, D: primer 420, E: primer 435, F: primer 494, G: primer 908, H: primer 948, I: primer 954, J: primer 989, K: primer 1201, L: primer 1214). Note: In each electrophoretic pattern, 1 to 5 are DNA from 5 different *Camellia* oil (DSX01, XTY02, WJ0079, F313, GC3171), respectively. 6 is DNA from *C. oleifera* leaf. M represents DNA Marker.

3. Results

3.1. Fatty acid composition overlapping limits the accurate adulteration identification of ***Camellia*** *oil*

The *Camellia* oil and other seven vegetable oils were analyzed by GC (Fig. 1) and their fatty acid compositions were compared (Table 2). The content order of fatty acids in palm oil from high to low is palmitic acid (45.137%), oleic acid (39.734%) and linoleic acid (8.742%). The main fatty acids of rapeseed oil are oleic acid (65.283%), linoleic acid (19.531%) and linolenic acid (5.748%). Most of the fatty acids in olive oil are oleic acid (76.129%), palmitic acid (11.713%) and linoleic acid (6.950%). The fatty acid composition of soybean oil is linoleic acid (54.580%), oleic acid (23.471%), palmitic acid (10.673%) and other fatty acids. The rich fatty acids in peanut oil were oleic acid (43.526%), linoleic acid (36.749%) and palmitic acid (10.711%). The arachidic acid (1.257%) and behenic acid (2.210%) in peanut oil are higher than that of other vegetable oils. The main fatty acids in maize oil are linoleic acid (54.032%), oleic acid (30.332%) and palmitic acid (12.202%). Most of the fatty acids in sunflower seed oil are linoleic acid (57.545%), oleic acid (30.220%) and palmitic acid (6.134%). The fatty acid composition of *Camellia* oil is oleic acid (80.150%), palmitic acid (8.432%), linoleic acid (8.391%) and other fatty acids.

From the composition point of view, there is not significant different between the fatty acid of Camellia oil and other vegetable oil, especially the oleic acid of *Camellia* oil is close to that of olive oil, and the content of palmitic acid of *Camellia*

Table 5 Sequence of primers for DNA of seven oil plants.

Plant/tissue used	Primer	Sequence
Oil palm/fruit	YZ-F	AATGCAAGGAGTACGAGTCA
	YZ-R	TTTAGAAGGGTAGCAGGTCA
Rape/seed	YZ-F	GATGACGCTGCTTTTTTCTC
	YZ-R	TTCTCACGCCTCTGTAATGC
Olive/leaf	YGL-F	CATTGGACGGTTCTTTGGCG
	YGL-R	TTTGGAGGTCTTGGGTGGGG
Soybean/seed	DD-F	CAAAACACAGGGCAGATTA
	DD-R	AGGTGCTTAGTTGGTAGGA
Peanut/seed	HS-F	GAAAACGAAAAATGAGGACG
	HS-R	ATGCAGAGAGGGAAGAAACA
Maize/seed	YM-F	GTTCTACACAAAACCCTCT
	YM-R	TTAAACTCTATGACCCTCC
Sunflower/seed	KHZ-F	GGATTATGGGAGTGTGTGA
	KHZ-R	GACCTTAGGATTGGTGTTG

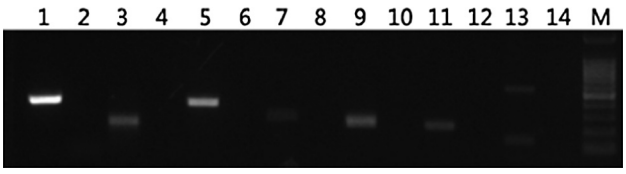

Fig. 3 Verification results of DNA extraction from seven oil plants. Note: 1, 3, 5, 7, 9, 11, 13 are the DNAs of sunflower, soybean, peanut, maize, rape, palm, olive, respectively. 2, 4, 6, 8, 10, 12, 14 are the negative control of each primer. M is DNA Marker.

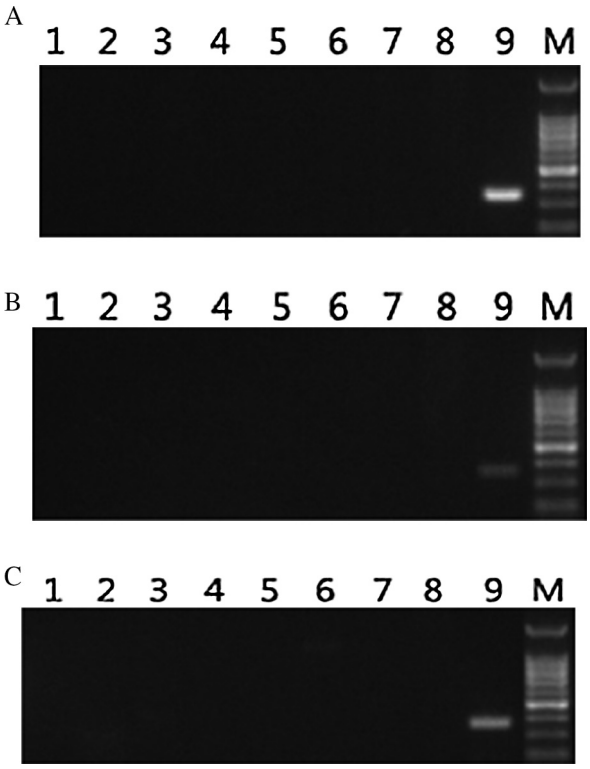

Fig. 4 PCR results of *Camellia* oil DNA-specific primers by exclusion screening (A: primer 124, B: primer142, C: primer 1214). Note: In this figure 1 is negative control of each primer. 2 to 8 are the DNAs of oil palm, rape, olive, soybean, peanut, maize and sunflower, respectively. 9 is the DNA of *C. oleifera* leaf. M is DNA Marker.

oil is as low as that of rapeseed oil and sunflower seed oil. Therefore, several kinds of oils can be mixed in a certain proportion to achieve similar fatty acid composition as *Camellia* oil. In the same way, flavor substances can be added to mixed oil so that the flavor is similar with *Camellia* oil. Hence, it is unable to accurately identify whether *Camellia* oil is adulterated depending only on the composition of fatty acids and flavor substances to determine.

3.2. Extraction of trace DNA from Camellia oil

DNA samples (10 μl) extracted by three methods were added into 1990 μl TE, and their concentrations and purities were determined by the nucleic acid protein analyzer.

It was observed that the modified SDS method has better effect extracting DNA from *Camellia* oil than other methods (Table 3). Moreover, the modified SDS method also has the advantages of short extraction time, small reagent toxicity and simple operation. Therefore, the modified SDS method is more suitable to extract *Camellia* oil DNA.

3.3. Screening of specific primers for Camellia oleifera-specific DNA

According to the *C. oleifera* EST library, 121 target genes without homology with the existing DNAs in GenBank were screened, and only 116 pairs of primers were successfully designed, which were synthesized by Beijing Genomics Institute. Each pair of primers was determined by 5 DNAs from different *Camellia* oil, which was positively controlled by DNA from *C. oleifera* leaf. Finally, only 12 pairs of primers were successfully screened out (Table 4) (Fig. 2).

Table 6 Sequences of specific primers and probe for qPCR.

	Primer name	Sequence
Primers	Q1214-F	GAGGAAACAGCAGCGGCAAA
	Q1214-R	GGTCGCAGCATTCGTCAAGG
Probe	P1214	TCGCTGTGCCTGCTGCGCCATGT

3.4. Exclusive screening of candidate Camellia oil DNA-specific primers

DNAs of seven oil plants (oil palm, rape, olive, soybean, peanut, maize, sunflower) were extracted by the modified SDS method. Seven pairs of primers were designed according to unique CDS sequences of these seven oil plants to verify if their DNAs were successfully extracted (Table 5). It was observed that DNA of each oil plant was extracted successfully (Fig. 3), avoiding false negativity during primer exclusive screening. It was showed that only 3 pairs of primers can bring specific DNA fragments by using *Camellia* oil DNA as template, but cannot bring any fragment by using DNAs of seven

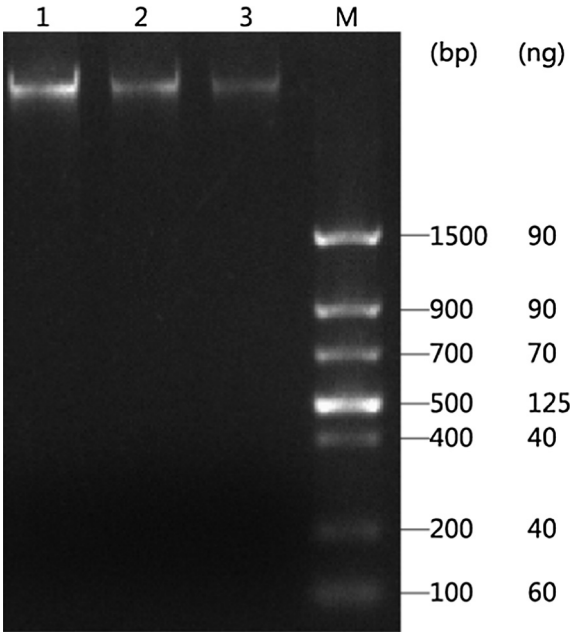

Fig. 5 Concentration quantitation of DNA from *C. oleifera* leaf. Note: 1 to 3 are 2.0 μl DNA, 1.0 μl DNA, 0.5 μl DNA, respectively. M is DNA Marker.

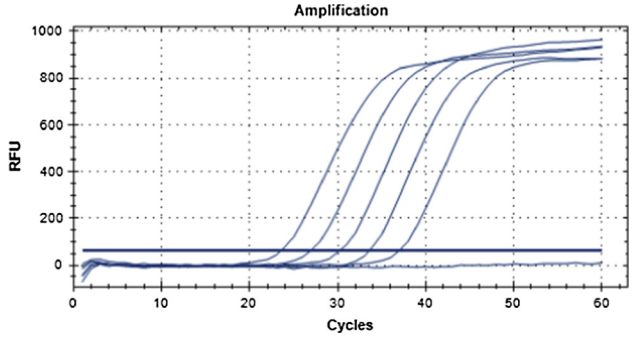

Fig. 6 qPCR result of gradient concentration of *C. oleifera* leaf DNA. Note: Curves from left to right are 100%, 10%, 1%, 0.1%, 0.01% concentration of target DNA from *C. oleifera* leaf. The curve below the threshold line is negative control.

vegetable oils as templates (Fig. 4). Therefore, only 3 pairs of *Camellia* oil DNA-specific primers were selected by exclusive screening.

3.5. Concentration regression curve of Camellia oil DNA

3.5.1. Design of specific qPCR primer and probe

According to above results, the primer 1214 was selected as final specific primer for the PCR, and then the corresponding primers and probe for qPCR were designed (Table 6). The target fragments brought by qPCR primer and probe were included in the fragments brought by the PCR primer 1214.

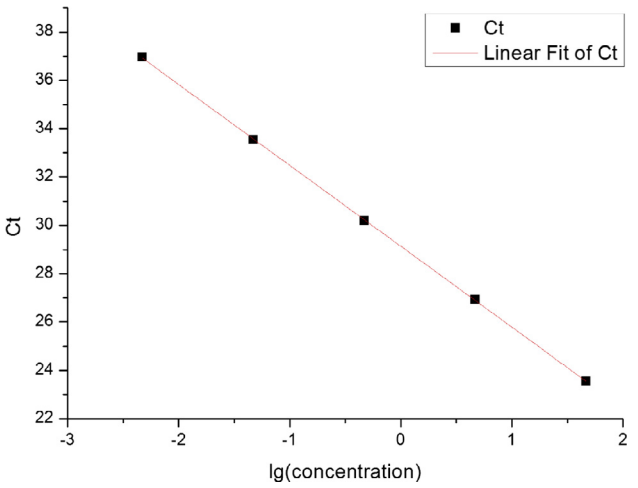

Fig. 7 Concentration regression curve of DNA from *C. oleifera* leaf.

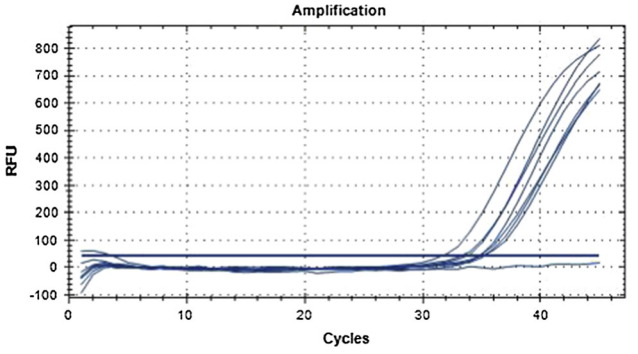

Fig. 8 qPCR result of DNA from different contents of *Camellia* oil. Note: Curves from left to right are 100%, 80%, 60%, 40%, 20% DNA concentration of ZZ003. The curve below the threshold line is negative control.

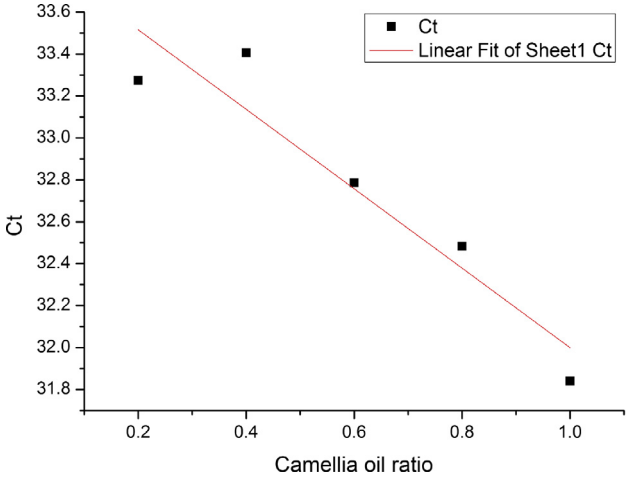

Fig. 9 Regression curve between Ct value and *Camellia* oil ratio.

3.5.2. Concentration regression curve of Camellia oil DNA basing on qPCR

2.0 μl, 1.0 μl and 0.5 μl DNAs of *C. oleifera* leaf were mixed with 10×DNA Buffer to 5 μl, respectively, which were checked by 1.5% agarose gel for DNA quantitation (Fig. 5).

Quantitative analysis of DNA was performed by the Volume Analysis component in the Quantity one software (Bio-Rad, USA), and showed that the concentration of target DNA from C. oleifera leaf was 47.05 ng/μl.

The target DNA was performed a gradient-dilution (100%, 10%, 1%, 0.1%, and 0.01%), which was used for qPCR accompanied with negative contrast (Fig. 6).

According to the principle of qPCR, the Ct value was linearly related to the DNA concentration, and the regression curve was achieved as: $y = -3.347x + 29.136$, $R^2 = 0.9999$ (Fig. 7).

3.6. Identification model of simulated Camellia oil adulteration

In order to simulate the adulteration of *Camellia* oil, the DNA of *Camellia* oil from sample ZZ003 was mixed with maize oil making *Camellia* oil ratio into 20%, 40%, 60%, 80% and 100%, which were determined by qPCR accompanied with negative contrast (Fig. 8). The linear regression curve between Ct value and *Camellia* oil content was achieved as: $y = -1.8\,95x + 33.895$, $R^2 = 0.8948$ (Fig. 9).

By simulating, two adulterated oils with 65% and 35% *Camellia* oil ratio were used to verify the regression curve by qPCR. The Ct value of adulterated oil with 65% *Camellia* oil ratio was 32.93, and the difference value was only 0.27 compared with the calculated result 32.66 in the fitting curve. The Ct value of adulterated oil with 35% *Camellia* oil ratio was 33.02, and the difference value was 0.21 compared with the calculated result 33.23 in the fitting curve. It was showed that the curve has high accuracy for little difference between detected value and theoretical calculated value, suggesting that this regression curve between Ct value and *Camellia* oil ratio could be efficient in identifying the adulterated *Camellia* oil basing on *C. oleifera*-specific DNA.

4. Discussion

Through determination of fatty acids in edible vegetable oil by GC, oleic acid and linoleic acid are the main fatty acid components of *Camellia* oil. However, the composition of fatty acids in *Camellia* oil is not special, and a mixed oil by a certain proportion of edible oils can also form a fatty acid composition close to that of *Camellia* oil. Therefore, adulteration identification of *Camellia* oil by fatty acids and flavor substances is still not accurate.

Identification of adulterated *Camellia* oil basing on *C. oleifera*-specific DNA can avoid the interference of homologous substances, and can accurately reflect whether the target *Camellia* oil is mixed with other edible oils, meanwhile, the *Camellia* oil ratio in the possible adulterated oil can be also confirmed. The current method only via fatty acid composition cannot determine the ratio of *Camellia* oil in target oil.

However, the new method of identifying adulterated *Camellia* oil basing on *C. oleifera*-specific DNA cannot reveal the type and ratio of the adulterated oils in target oil. So in the posterior researches, the efficient determination of heterologous DNA from other oils in target oil should be addressed firstly, then identification of the kinds and types of possible adulterated oils should be developed.

5. Conclusions

Generally, a new approach was developed to identify *Camellia* oil adulteration basing on *C. oleifera*-specific DNA. First of all, primers 1214 (F/R) had high efficiency and specificity, and was successfully used in identification of adulterated *Camellia* oil. Fluorescence qPCR primers Q1214 (F/R) and TaqMan probe P1214 were successfully used to measure DNA concentration and *Camellia* oil ratio by qPCR. Finally, the mathematical model between DNA ratio and Ct value was established to identify *Camellia* oil adulteration.

Acknowledgement

The authors acknowledge the financial support from the key science and technology project of Hunan Food and Drug Administration, China (R201514), the science and technology project of Hunan Province, China (2016NK2154), and the National Natural Science Foundation of China (31172257).

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