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Influence of microwave application on DNA degradation in mildly and highly processed orange

Farzaneh Abdolmaleki, Mahnaz Mazaheri Assadi*, Hamid Ezzatpanah and Masoud Honarvar

Food Science and Technology Department, Faculty of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, Tehran, Iran

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ABSTRACT

Isolation of high yield and quality of genomic DNA is paramount for ensuring confidence in molecular analyzing food. PCR-based methods are the most common and reliable methods developed thus far. We developed genomic DNA extraction methods and also assessed the effect of Microwave application on DNA degradation in pasteurization and drying of orange samples. The purity and concentration of DNA obtained from the samples were evaluated by spectrophotometric and gel electrophoresis analysis. It was found that the quantity and quality of DNA by CTAB (cetyltrimethylammonium bromide) method were higher than by Wizard kit method. The results confirm that the stability of DNA in orange products differed in terms of processing conditions. Microwave heating as compared with thermal heating resulted in lower DNA degradations and also drying process led to the most severe changes in contrast to pasteurization process in orange samples.

Keywords: Microwave heating, Orange juice, Dried orange, Degradation,

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INTRODUCTION

Citrus fruit as a nourishing fruits most widely consumed worldwide. They are a great source of nutritionally important compounds such as vitamin C and fibber as well as in flavonoids and terpenes [1, 2]. In recent years, the fresh consumption of the citrus fruits having decreased. Although, the demand for citrus derived products as a healthy, natural and tasty source is continuously increasing. Heat processing of orange products is essential for its shelf life, which decrease organoleptic quality[3].

The use of microwave heating for processing materials has the potential to increase product yields, reduce processing time and promotes product purities by decreasing unwanted side reactions compared to conventional heat transfer. Anyway, some critical points arose when performing MW heating, for instance, difficulties were encountered in temperature readout and control whereas traditional probes fail [4].In such a connection, a number of studies have been conducted to improve microwave drying by investigation the effects of different power levels and drying temperatures[5-9]. Zhang et al. (2006) have reviewed recent research on microwave assisted drying, focusing primarily on fruits and vegetables [10].

The product can be traced at any point of its travel along the food supply chain. Different genetic and biology-based methods exist for this purpose[11]. All these methods are based on nucleic acid analysis[12].This is accomplished by using either the DNA itself or its proteins[13-16]. An example for the former group of methods is Polymerase Chain Reaction (PCR), which identifies the novel DNA and makes it possible to detect any traces of genomic DNA remaining in a food matrix or combined with contaminants [17].On the other hand, a major protein-based

identification method is the enzyme-linked immune sorbent assay (ELISA), which identifies the novel protein in raw products [13, 18-20]

In the molecular analyses of food matrices, Peano et al. (2004)determined that three different critical points, such as the degree of DNA damage(e.g., depurination of the DNA), the presence of PCR inhibitors, and the short average length of DNA fragments have been regarded. These parameters are influenced by the samples itself and also the physical and chemical treatments performed during the production of the food [21].Bauer(2003) confirmed that various factors totally as: shear forces, temperature, pH, ionic strength, chemical agents, and enzymes affect the primary structure of DNA, so that its decay leads to depurination, deamination, and strand breaks, thereby increasing sensitivity to further degradation[22, 23].

Conventional PCR used as the main analytical tool for most studies on DNA degradation and employed as a qualitative method to determine the processing steps, which led to the loss of target molecules [24]. Chen et al. (2007) used a conventional PCR method to analyze the impact of processing procedures, such asjordaning, syruping, blending, homogenization, and sterilization during soymilk preparation from Roundup Ready Soybean[25]. Furthermore Vijayakumar et al. (2009) detected that amplicon size of gene sequences in MON-810 corn and Roundup Ready soybean during conventional PCR method was affected by heat processing viz. microwaving and autoclaving [26].

However, studies about the effect of microwave treatments on DNA degradation of processed orange are very limited. Therefore, the objectives of this studyare to evaluate and compare the effects of microwave heating and traditional heating on orange DNA degradation in addition to the effect of different treatments (drying and pasteurization) on the quantity and quality of the extracted DNA. We also aim to compare two methods of DNA extraction (CTAB protocol and Wizard kit) in terms of their efficiency.

MATERIALS AND METHODS

1. Plant materials

Orange fruits (*Citrus sinensis* L., var. Valencia late) were purchased from a local supermarket and stored at 5ºC for a maximum of two days until the experiments were carried out. After suitable washing and hygienization, oranges (approximately a total of 40 kg) were divided into five similar groups and then separately processed according to different procedures:

a. The fruits were sliced (5 mm thick).

b. The fruits were sliced (5 mm thick), and the blanching process was carried out at 80 ºC for 6 min (this pre-treatment inactivated the enzymes). Drying experiments were carried out in a microwave oven. The incident and reflected power of MW were controlled by using a directional coupler in the power measure system to estimate the power absorbed by sample. 300 W of supplied MW power were applied. In each experiment, 5 slices were put in the oven.

c. The fruits were sliced (5 mm thick). After blanching process at 80 ºC for 6 min., the samples were dried in hot air drier. The temperature and velocity of the air were 60 ºC and 2 m/s, respectively.

d. The oranges were half cut and squeezed using a domestic squeezer (Lomi mod. 4, Madrid, Spain), and then was filtered using a 2 mm steel sieves. The juice was immediately pasteurized between 95 and 105 ºC for 10 s.

e. The oranges were squeezed and then pasteurized in a domestic microwave oven (Panasonic 600 W power) for 30s.

2. DNA isolation

Genomic DNA was extracted from 200 mg of fresh oranges and dried oranges. Samples were placed in liquid nitrogen before being ground into powder. For pasteurized orange juice, genomic DNA was isolated from 3ml of the sample after centrifugation at 14,000 rpm for 5 min and separation of pellets. CTAB method and Wizard kit were used to extract genomic DNA from raw and processed orange products.

2.1. CTAB method

This procedure is a modification of the protocol indicated in (1), carried out following the indications of Doyle and Doyle (1990) [27].

Reagents: Extraction buffer [20 g/L CTAB(Sigma), 1.4 mol/L NaCl, 0.1 mol/L Tris–HCl, 0.02 mol/L Na2EDTA pH 8.0]; Chloroform; Isopropanol; Ethanol 70%; 0.1X TE buffer [10 mMTris–HCl, 1 mM EDTA pH 8]; RNaseA enzyme.

Protocol: 500µl of extraction buffer was preheated at 65 °C for 20-30 min and with 40 µL solution of proteinase K was transferred into a 1.5-ml Eppendorf tube containing ground tissue of each sample. Tubes were incubated at 60 °C for at least 1 hour and were shaken gently every 5 min. After centrifugation at 14000 g for 5 min at room temperature, the aqueous phase was transferred to a new eppendorf, mixed with 60 µl Chloroform. The sample tubes were centrifuged at 14000 g for 10 min, then the supernatant was collected, 50 µl isopropanol were added, the mixture was shaken. After 30 min of incubation at room temperature, the mixtures were centrifuged at 14000 g for 15 min. The pellet was washed twice with 250 ml of cold ethanol, left to dry at room temperature before it was dissolved in 20 ml of TE buffer with RNase (200 ng/µL) then incubated at 65 ºC for 2 min. The collected DNA was ready for use or stored in a freezer at -20 ºC.

2.2.Wizard kit
DNA isolation from raw and processed orange was carried out with commercial kit according to manufacture᾽s instructions.

Reagents: Extraction buffer[10 Mm Tris–OH, 150 mM NaCl, 2 mM EDTA pH 8, 1% (w/v) SDS]; guanidine hydrochloride (5 M); proteinase K solution (20mg/ml); Wizardresin (Promega, Madison, USA); elution buffer (10 mMTris-OH).

Protocol: 860µL of extraction buffer (preheated at 65 °C),100 µL of guanidine hydrochloride and 40 µL solution of proteinase K were added to fine powder of each sample in a 1.5 mL microtube. The mixture was vortexed and after incubation at 65 ºC for 80 min,was vigorously shaken and centrifuged at 13,500 g for 10 min. Following this, 500 µL of the upper phase was transferred to a new tube, mixed with Wizardresin and pushed through a Wizard minicolumn (Promega, Madison, USA). Thecolumn was washed with 2 ml of isopropanol then was spun at 12,000 g for 5 min. The Nucleic acid was eluted with 50 µL ofelution buffer(preheated at 65 °C) and after incubation at room temperature for 1 min, column was centrifuged at 10,000 g for 2 min. Finally, the achieved DNA was stored in a freezer at -20 ºC

3. Quantification of genomic DNA
Quantity of extracted DNA was assessed by measuring spectrophotometric optical densities (OD) at 260 nm and 280 nm [28]. The purity of genomic DNA was measured using the UV absorbance ratio of 260 and 280 nm. For each sample, concentrations (ng/µL) were evaluated by measuring the absorbance at 260 nm [29].

4. Agarose gel electrophoresis analysis
The quality of genomic DNA were analyzed by gel electrophoresis using 0.7% (w/v) agarose gel (TAE buffer system)contained with superior Gel Red 1x (Biotium, Hayward, CA, USA: 0.5 µg/mL) [28]. The marker (1 kb DNA ladder, Promega, Madison, USA) was used to determine the DNA fragments' size. Then gel was visualized under UV light by employing a Bio-Rad Gel Doc 2000 (Bio-Rad Laboratories Inc., Hercules, CA).

5. PCR amplification
primer sequences and expected amplicon lengths are listed in Table1.

Table1- List of PCR primers used

Target gene	Primer	Sequence(5´-3´)	Amplicon size (bp)
GAPC2(Glyceraldehyde-3-Phosphate Dehydrogenase C2)	GAPC2 for GAPC2 rev	TCTTGCCTGCTTTGAATGGA TGTGAGGTCAACCACTGCGACAT	950bp
β-tubulin	β-tubulin for β-tubulin rev	TTCCCATCTCCTAAGGTCTCTG CCAAAGCTTGGAGTGCTGAG	340bp
18S rRNA	18S rRNA for 18S rRNA rev	GATGAAGAACGTAGCGAAATGC GGGTAATCCGCCTGACC	200bp

Primer Express™ 2.0 software (Applied Biosystems, Foster City, CA, USA) was used to design primers for PCR analysis. Primers synthesized by Sigma Genosys (Sigma-Aldrich, UK) were diluted to a final concentration of 10 pmol µl−1 with Milli-Q water and stored at -20 ◦C until used.

Amplifications were performed using a TC-512 thermal cycler (Techne Inc. USA).The reaction mixture (25 mL) consisted of 12.5 µL of TaqMan-Universal PCR Master Mix (Life Technologies), 1µL of primers with final

concentration of 0.4 µM each and 100 ng of DNA template. The thermal profiles employed in PCR analysis were as follows:

• For GAPC2: denaturation for 2 min at 94°C; amplification for30 s at 94°C, for 45 s at 54°C, for 60 s at 72°C; number of cycles36; final extension for 5 min at 72°C.
• For β-tubulin: denaturation for 2 min at 94°C; amplification for30 s at 94°C, for 30 s at 55°C, for 30 s at 72 °C; number of cycles25; final extension for 7 min at 72°C.
•For 18S rRNA: denaturation for 2 min at 94°C; amplification for30 s at 95°C, for 40 s at 54°C, for 40 s at 72°C; number of cycles40; final extension for 3 min at 72°C.

PCR products were electrophoresed through a 1.7% agarose gel containing superior Gel Red 1x (Biotium, Hayward, CA, USA: 0.5 µg/mL), .As a size reference, a DNA ladder (100bp DNA ladder, Promega, Madison, USA) was employed and Visualization of the gels was performed with a Bio-Rad Gel Doc 2000 (Bio-Rad Laboratories Inc., Hercules, CA).

6. Statistical analysis
All experiments were performed in triplicates. The significance of differences among the different treatments was determined with analysis of variance (ANOVA) and two-tailed independent-sample T-test in SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was applied. To approve the statistical significance of all data, the values of means ± S.D (standard deviation) were reported.

RESULTS AND DISCUSSION

This study compared two methods of extracting DNA from fresh and processed orange in an attempt to determine the purity, concentration, and quality of the DNA samples.

1. The purity, concentration, and quality of DNA extractions
The purity and concentration of DNA solution samples are presented in Table 1. DNA samples extracted from: fresh orange, orange juice pasteurized with traditional and microwave heating, and orange slice dried with traditional and microwave heating.

Table 2:The purity and concenteration of genomic DNA solutions extracted from orange samples using two extraction methods

	Products	CTAB method	Wizard
Purity	Fresh orange	$1.99{\pm}0.05^{a}$	$1.89{\pm}0.06^{a}$
	MW pasturized orange juice	$1.92{\pm}0.01^{ab}$	$1.84{\pm}0.04^{ab}$
	Thermal pasturized orange juice	$1.87{\pm}0.05^{b}$	$1.72{\pm}0.07^{b}$
	MW dried orange slice	$1.75{\pm}0.05^{c}$	$1.83{\pm}0.04^{ab}$
	Thermal dried orange slice	$1.72{\pm}0.05^{c}$	$1.75{\pm}0.07^{b}$
Concenteration (ng/µL)	Fresh orange	$127.7{\pm}4.5^{a}$	$108.7{\pm}6.5^{a}$
	MW pasturized orange juice	$100.7{\pm}8.0^{b}$	$81.0{\pm}3.0^{b}$
	Thermal pasturized orange juice	$84.0{\pm}3.0^{c}$	$56.7{\pm}1.5^{c}$
	MW dried orange slice	$37.3{\pm}2.0^{d}$	$28.0{\pm}1.0^{d}$
	Thermal dried orange slice	$15.13{\pm}0.5^{e}$	$11.7{\pm}1.5^{e}$

Data are means ± S.D of triplicate measurements
Values with different superscript upper case letters in a column are statistically significant at P < 0.01.

According to the information in Table 1, A260nm/A280nm ratios of the DNA solutions extracted from orange product samples via CTAB modified with an average of 1.85 were significantly higher(P< 0.05) than the Wizard kit. Therefore, the genomic DNA extractions with more purity from orange samples were obtained, using the CTAB protocol. Although, the absorbance ratios of DNA extracted by Wizrd kit procedures were not within the range of 1.9–2.0.These results indicating that DNA extracts obtained with CTAB procedure were relatively pure and contained low amounts of proteins, RNA, or other contaminants.

The results in Table 1 suggest that the concentrations of the intact DNA in fresh orange and processed orange samples by CTAB method were in the range 73 ng/µL. However, those by Wizard kit method were in the range 57ng/µL. In other words, the yields of the genomic DNA in orange samples using the Wizard kit were significantly lower (P< 0.01) than the CTAB method. It is also evident that the DNA extraction kits are generally faster than CTAB methods; however, they may be more expensive.

In this context, an appropriate amount of DNA could be obtained from orange products even from highly processed fruit products. Greiner &Konietzny(2008) by employing the CTAB and Promega Wizard kit protocols extracted

appropriate quantity of DNA from processed foods[30]. Our results were also consistent with the results of other previous studies [31, 32]. Although some researches notified that the difficulty of extracting sufficient amount of genomic DNA from processed food related to the influence of processing treatment as; heat, low pH and pressure [21, 26, 33, 34 and 35].

The effect of pasteurization and drying on the purity and concentration of extracted DNA was also investigated. The results (Table 2) confirm that the concentration and yield of intact DNA extracted from unprocessed orange was higher than the quantity of DNA extracted from processed orange samples employing two extraction protocols. However, results demonstrated that the average yield of DNA from orange juice, pasteurized by traditional heating, were significantly lower than those pasteurized by microwave heating.

In all DNA extraction procedures tested, the yield and concentration of processed oranges were significantly lower than unprocessed fruits, which possibly indicating the relation of breaks in DNA strands. Peano et al. (2004) showed that physical and chemical treatments, such as heat, could result in fragmentation of genomic DNA strands and also decreased the average fragment size [21]. Although, Gryson et al. (2007) confirmed that heat processing reduces the overall DNA fragment length without reducing the DNA concentration [36].

Moreover, Turci et al. (2010) illustrated the effect of temperature as a major factor on DNA depurination. They concluded that temperature can cause enormous cross-linking compounds, resulting in ball-shaped nucleic acids and proteins which hamper with nucleic acid extraction and/or amplification [37]. Furthermore, Gryson (2010) analyzed transgenic soybean (soybean sauce, soymilk, sufu, tofu, and notto) and transgenic corn (corn puff snacks, corn chip snacks, and corn flakes). He found that any chemical or physical treatment of food samples would affect the removal or damage of the extracted nucleic acid[38].

The genomic DNA extracted from fresh and processed oranges, using the two experimental methods, were run on agarose gel in order to evaluate the quality of the extracts. Fig. 1 shows the results from gel electrophoresis.

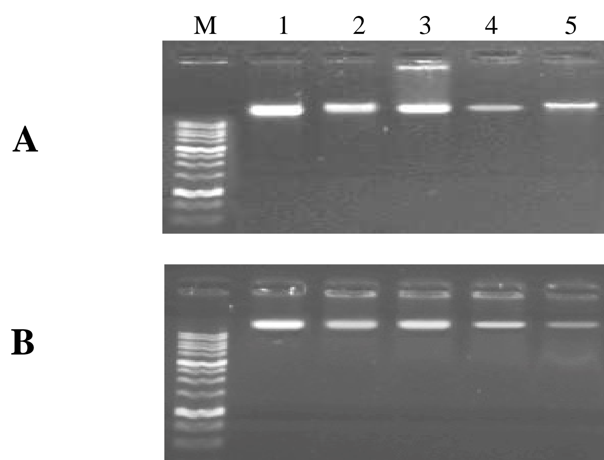

Fig. 1.Agarose gel electrophoresis of genomic DNA obtained from processed orange samples
A, CTAB method; B,Wizard kit: Lane M, 1kb ladder size marker; Lane 1,Fresh orange; Lane 2, MW pasteurized orange ; Lane 3, thermal pasteurized orange; Lane 4, MW dried orange; Lane 5,thermaldried orange.

It can be seen that the genomic DNA extracted from apple via the modified CTAB method are free from smears and impurities so that after running on agarose gel, they appeared in a sharp, pure, and with the highest degree of DNA quality(Fig. 1A, lanes 1-5).

The analysis of gel electrophoresis confirmed that a strong correlation exists between the efficiency of DNA extraction method and the quantity of orange DNA and also there is a correlation between the degree of processing and recovery of genomic DNA. Food processing condition, such as freezing, high temperature, and pressure have drastic impact on DNA fragmentation.

2. PCR amplification

PCR analysis was carried out on the extracted DNA which had adequate quality and quantity; that is, on the extracted DNA using the CTAB methods. In order to assess the degree of DNA degradation on PCR amplification, extracted DNA from orange samples were amplified targeting sequence of GAPC2, β-tubulin and 18S rRNA.

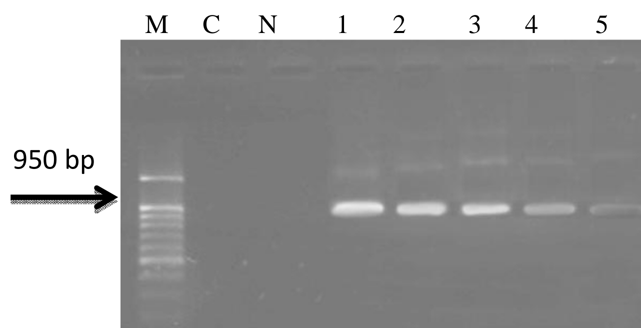

Fig. 2. Agarose gel electrophoresis of PCR amplification of DNA extracted from orange samples usingGAPC2 gene
Lane M: 100pb ladder size marker; Lane C, premise control; Lane N, non-orange-fruit;Lane 1,Fresh orange; Lane 2, MW pasteurized orange ; Lane 3, thermal pasteurized orange; Lane 4, MW dried orange; Lane 5,thermaldried orange.

The results (Fig.2) of Agarose gel electrophoresis, performed on the PCR amplification of extracted DNA, obtained from orange samples, using GAPC2 gene.

In this study, for all fruit products, the 950bp fragment was observed in the agarose gel electrophoresis. It was found that the drying process had the most influence on GAPC2gene and microwave heating resulted in the lower changes than traditional heating.

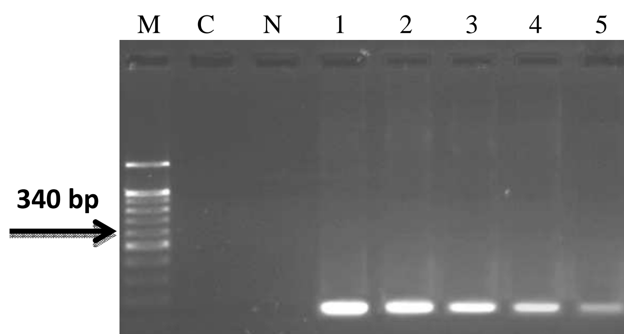

Fig. 3.Agarose gel electrophoresis of PCR amplification of DNA extracted from orange samples using β-tubulin gene
Lane M: 100pb ladder size marker; Lane C, premise control; Lane N, non-orange-fruit;Lane 1,Fresh orange; Lane 2, MW pasteurized orange ; Lane 3, thermal pasteurized orange; Lane 4, MW dried orange; Lane 5,thermaldried orange.

Fig. 3 presents the PCR detection results of β-tubulingene in orange and derived products. Detection results of the 340bp fragment of β-tubulingene show that this gene had not been degraded for fresh fruit. However, for dried orange, sharp bands were not observed in the agarose gel electrophoresis. The β-tubulingene, affected of different methods of processing fruit, was more stable thanGAPC2 gene.

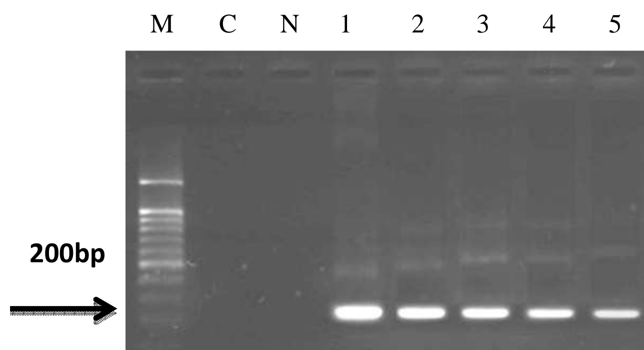

Fig. 4.Agarose gel electrophoresis of PCR amplification of DNA extracted from orange samples using18S rRNAgene
Lane M: 100pb ladder size marker; Lane C, premise control; Lane N, non-orange-fruit;Lane 1,Fresh orange; Lane 2, MW pasteurized orange ; Lane 3, thermal pasteurized orange; Lane 4, MW dried orange; Lane 5,thermaldried orange .

Fig. 4 shows the results of PCR amplification of 18S rRNAgene in orange samples which compares the quality of recovered amplifiable genomic DNA after production of dried and pasteurized samples with both traditional and

microwave heating. Detection results of the 200bp fragment of 18S rRNAgene show that this fragment had not been degraded for fresh fruit. However, for dried orange, sharp bands were not observed in the agarose gel electrophoresis.

In current study, our findings demonstrated that 18S rRNA primer with amplify fragments of 200 bp had the best result PCR analysis. It was previously recommended by Hupfer et al. (1999) [39].The result of Vijayakumar et al. (2009) showed that recovery and amplicon size <200 bp are most suitable for the successful detection of GMO when analyzed soybean and maize[26]. Although Costa et al. (2010) reported that target DNA fragments of ≤ 100 bp were better strategies for detecting nucleic acid in vegetable oils[40]. Several researchers have showed that the choice of small DNA fragment is critical for analyzing processed food[26, 40- 43].

The results of PCR amplification of three genes illustrated that, the severity of damages employed in fruit processing have a great influence on the quality of the recovered amplifiable DNA. The present study confirms that drying and pasteurization process have a significant impact on 950bp, 340bp and 200bp fragments.

It is also evident that the DNA yield from fresh fruit was nearly 3.5 times greater than dried oranges. This could be due to drying process included two procedures as blanching treatment and intensity of heat, resulted in the most severe DNA degradations. Moreover, the effect of microwave resulted in lower degradation than pasteurization of fruit juice. Similar trends were previously reported by Vijayakumar et al. (2009) who mentioned amplicon sizes of gene sequences in MON-810 corn and Roundup Ready soybean during conventional PCR method was influenced by heat processing *viz*. autoclaving and microwaving[26].

In current study, the pasteurization process on samples of fruit juice led to slight nucleic acid degradations. Our finding was also in agreement with those reported by Wiess et al. (2007) who analyzed the effect of pasteurization conditions on plant DNA degradation in the food matrix[44].

By making a comparison between fig 2,3 and 4, the presence of clear and sharp band without any extra bands in amplification of 18S rRNA for orange samples, it is proved that in PCR amplification 18S rRNA fragment is the most stable. This finding was in agreement by other researchers [32, 45 and 46].

Based on these results, we conclude that convectional PCR with three primers for analyzing DNA degradation is satisfactory, even in highly processed orange products. Although, this work could be extended by employing Real time-PCR and more primer for better analysis in future.

CONCLUSION

We evaluated two different procedures to obtain a high DNA yield from fresh orange and processed orange products (dried sliced orange, pasteurized orange juice). The extraction of DNA from orange and processed products was successfully carried out with the procedures of CTAB method. Subsequently, results demonstrate that microwave treatment of orange samples had significantly lower effect on DNA degradation, when compared with traditional heating, also fruit drying with a longer heating time was more severe process than fruit juice pasteurizing procedure.

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