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Identification of Safflower as a fraud in commercial Saffron using RAPD/SCAR marker

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Abstract

Saffron (*Crocus sativus*) is the most valuable and indigenous crop in Iran. The stigmas of flower are used as a popular natural flavouring, colouring and medicinal agent. However, the market suffers from frauds in this plant such as mixing with safflower petals due to high profit. Identification of these frauds with conventional and biochemical methods is difficult and low sensitive. Therefore, application of molecular markers such as random amplified polymorphic DNA (RAPD)/sequence characterized amplified regions (SCAR) is being considered as an alternative. In this study, DNA was extracted from dry stigmas of 5 Saffron accessions and dry petals of 7 safflower cultivars. RAPD reactions with ten 15-mer random primers resulted in two specific monomorphic bands (500 and 700 bp) for safflower, while they were absent in saffron accessions. PCR analysis with specific SCAR primers amplified two specific bands (414 and 589 bp) for safflowers in different combinations of saffron stigmas and safflower petals. This was the case with very low rates or 1% of safflower. Therefore, this method seems to be suitable for fraud identification of safflower petals in commercial saffron samples.

***Keywords*:** fraud identification, RAPD/SCAR, safflower, Saffron

Introduction

Saffron as the most expensive agricultural product of the world has a special importance among the exported products of Iran. More than 80% of saffron production worldwide belongs to Iran (Trade Promotion Organization of Iran, 2009). Saffron is produced from dried stigmas of *Crocus sativus*, and is considered as the most expensive spice in the world (Amir Ghasemi, 2001). Each flower has three stigmas which weighted 5 mg, and, 200000 flowers must be carefully picked one by one in order to produce 1 kg spice (Kafi et al., 2003). So, its high value has made saffron the object of frequent adulteration, and also being the object of intense chemical and biotechnological research (Fernandez, 2004). Under the prevention of Food Adulterant Act, an adulterant is any material which is employed for the purposes of adulteration. Mixing of similar materials such as beet, pomegranate fibers, red-dyed silk fibers, the flowers of other plants such as *Carthamus tinctorius* or safflower, *Calendula officinalis* or marigold, arnica and tinted grasses are the most fraudulent activities in saffron (Kafi et al., 2003). The limitation of saffron production and also its high price caused some efforts for its artificial production and frauds in which one of the most common ways is adding safflower petals to saffron stigmas (Ghasemi, 2001). There are different ways for determining adulteration in saffron such as chemical (Haghighi et al., 2007; Lage and Cantrell, 2009) and physical (Tsimidou and Tsatsaroni, 1993; Cuko et al., 2003; Zalacain et al., 2005) measurements but their sensitivity is usually low. Recently, progressing in DNA techniques makes it possible to identify any unwanted biological materials in plant products, especially in saffron (Pardo et al., 2003; Dnyaneshwar et al., 2006). PCR has a high potential for adulterant detection due to its simplicity, sensitivity, specificity as well as rapid processing time and low cost (Vidal et al., 2007; Mafra et al., 2008; Reid et al., 2006). The PCR-based methods used for adulterant detection and authentication include the amplification using species specific primers, DNA fingerprinting methods like RAPD (Williams et al., 1990), PCR with arbitrary primers (AP-PCR) (Welsh and McClelland, 1990), DNA amplification fingerprinting (DAF) (Caetano Anolles et al., 1991), Inter Simple Sequence Repeats (ISSR) (Zietkiewicz et al., 1994), PCR-RFLP (restriction fragment length polymorphism) (Konieczny and Ausubel, 1993). Among these, RAPD marker uses

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small and random primers. In order to solve the non-repeatability problem of RAPD marker and specific band identification, RAPD/SCAR marker was developed (Dnyaneshwar et al., 2006). Today, SCAR markers, made by RAPD sequences, are successfully used for both the identification of some crops such as grape (Vidal et al., 2000), olive (Hernandez et al., 2001; Dovwri et al., 2006) and the identification of frauds in other plants such as *Embelia ribes* (one of the important plants used in Indian traditional medicine) (Devaiah and Venkatasubramanian, 2008), *Phyllanthus emblica* (Dnyaneshwar et al. 2006), the fibers of bamboo (mainly used in the pulp, paper and charcoal industries) (Das et al., 2004), olive oil (Dovwri et al., 2006), commercial Pelargonium (Lesur et al. 2001), Korean ginseng (Park et al., 2006), poplar commercial clones (Fossati et al., 2005) and *Encephalartos* (the second largest genus of the cycads) (Prakash and Van Staden, 2008) and also for discrimination between Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Zhang et al., 2006). Devaiah and Venkatasubramanian (2008) used RAPD-SCAR marker for fraud identification in *Embelia ribes* which is the traditional pharmaceutical plant in India. In their study, RAPD reaction resulted in specific band (906 bp) for this species which was used for designing SCAR primer to detect this species among frauds. There are some reports about fraud identification and quality control in saffron by biochemical methods (Tsimidou and Tsatsaroni, 1993; Cuko et al., 2003; Haghighi et al., 2007; Lage and Cantrell, 2009; Maggi et al., 2011) but there is not any report of application of RAPD-SCAR method for fraud identification in saffron, specially about adding safflower petals. Lozano et al. (1999) used an HPLC method for simultaneous detection, identification and quantification of the secondary metabolites in commercial saffron and some possible artificial colorants. Regarding the sensitivity of molecular marker and the importance of fraud identification in saffron, we used RAPD/SCAR marker for identification of safflower frauds in commercial saffron.

Materials and Methods

Plant materials

Fresh leaf tissues and dry stigmas of saffron *(C. sativus)* samples or accessions were used in this experiment. They were collected from five different regions of Iran (Ghaen, Gonabad, Barakuh Gonabad, Torbat Heidariieh, and Science and Technology Park). Fresh leaf tissues and dry petals of seven safflower *(C. tinctorius)* varieties (IL-111, 2819, 279, K.W.3, K.W.6, K.W.16, 295), were provided from gene bank collection of Agricultural Research Center of Khorasan province, Iran.

RNA extraction and RAPD-PCR

DNA was isolated from fresh and dry as well as commercial samples of saffron and safflower using Modified Cetyl Trimethyl Ammonium Bromide (CTAB) extraction method (Saghai-Maroof et al, 1984). In brief, fresh leaf tissues (60-110 mg) or dried tissues (30-50 mg) were ground in liquid nitrogen. Freshly prepared extraction buffer (containing Tris-HCl 1M (pH=7.5), NaCl 2.5 M, EDTA 0.5 M (pH = 8), CTAB 2%, PVP 2%, and BME 1%) was added. The following steps were done according to the Modified CTAB procedure of Saghai-Maroof et al. (1984). PCR was done by ten 15-mer random primers (table 1) in a reaction mixture of 25 µl volume. Each reaction tube contained 50 ng DNA, 1 U Taq DNA polymerase enzyme, 200 µM of each dNTPs, 1x *Taq* DNA polymerase buffer, 4 mM $MgCl_2$ and 10 pmol of each primer. Amplifications were carried out in a DNA thermal cycler (Biometra) using following parameters: 94°C for 3 min; 35 cycles at 94°C for 1 min, annealing in 3 centigrade degrees below melting temperature for 1min, and 72°C for 1 min; and a final extension at 72°C for 5 min. PCR products were loaded on agarose gel (1.2%) electrophoresis in 0.5X TBE buffer.

Selecting of monomorphic bands and DNA fragment sequencing

Amplicons, which were monomorphic for all the safflower varieties but absent in five saffron samples were identified (figure 1). The putative markers amplified by the random primer RAP5, were excised from agarose gel with sterile gel slicer and purified using Clean Qiagen Gel Extraction kit. The A-tailed DNA was ligated into a TA-vector using Rapid DNA ligation kit (Roche, Germany). The ligated vector was introduced into competent *Escherichia coli* strain DH5α according to the protocol of transformation by calcium chloride. The transformed colonies were picked up from the LB medium with ampicillin as selective agent. Recombinant plasmids were isolated from each overnight grown colony with High Pure Plasmid Isolation kit (Fermentas, Germany). Confirmation of the clones was done by digesting the recombinant plasmid using *sacI* enzyme.

Recombinant plasmids were sequenced by automated sequencer in Macrogen Inc. of Korea. Based on the sequencing, some pairs of SCAR primers were designed. The SCAR primer pairs

were used for PCR amplifications of genomic DNA from the seven safflower varieties and five samples of saffron and also the DNA extracted from the mixed plant material of safflower in saffron with different combinations (1 %, 1.5 %, 2.5 %, 5 %, 7% and 10 %). PCR reaction was done according to the volumes and cycle program of RAPD reaction which mentioned before by chossing 58°C as annealing temperature. Homology searches were performed within GenBank's non redundant database using the BLAST program.

Results

Identification of RAPD marker for safflower

High molecular weight genomic DNA was isolated successfully from all the fresh and dried tissues. RAPD reactions resulted in some monomorphic bands in a few primers. Of them, RAP5 primer produced distinct and reproducible amplification profile for all the DNAs. Primer RAP5 consistently amplified two intense bands of 473 and 717 bp for all the safflower varieties, which were absent in the saffron samples (figure 1). These bands, named as SAF-L717 and SAF-L473, were selected as putative safflower specific markers.

The length of the SAF-L473 and SAF-L717 marker sequences were 473 and 717 bp, respectively. BLAST results revealed that the SAF-L473 sequence has 74% homology with mitochondrial ccb206 gene (for cytochrome C biogenesis protein with the accession number of AM183222.2) of *Helianthus annuus*. Our sequence was submitted to NCBI with the gene bank number of GU183488.1. The SAF-L717 sequence has no similarity with any sequence in NCBI database but shows certain levels of sequence-similarity with some plant nucleotide sequsences in EMBL database such as the yellow starthistle *Centaurea solstitialis* cDNA with the ID of EMBL-Bank: EH764694 (84% identity). The SAF-L717 sequence was submitted to the NCBI with the accession number of GU183487.1. There was however no similar sequences in safflower genome for these two sequences.

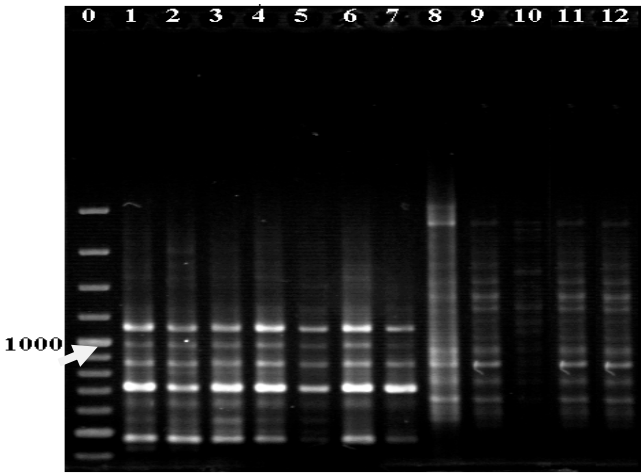

Figure 1. RAPD profiles of safflower varieties and saffron samples amplified with RAP5 on 1.2% agarose gel (0: Size marker (100 bp), lanes 1-7: Safflower varieties (1: IL-111, 2: 2819, 3: 279, 4:K.W.3, 5: K.W.6, 6: K.W.16, 7: 295), Lanes 8-12: Saffron samples; (8: Ghaen, 9: Gonabad, 10: Barakuh Gonabad, 11: Torbat Heidariieh, 12: Science and Technology Park)

Amplification Using SCAR Primers

Based on the sequencing, three pairs of SCAR oligonucleotide primers (SAF-L70 primer for SAF-L717 sequence, SAF-L40 and SAF-L4 for SAF-L473 sequence) (table 2) were designed by Primer3 online website which could amplify approximately 589, 414 and 412 bp of the genomic safflower DNA. The designed SCAR primer pairs were used to amplify genomic DNA from the 7 safflower varieties, while the DNA from the saffron specimens was not amplified by these primers. A single, distinct band of 414 bp was obtained from the DNA isolated from all the 7 safflower varieties and no non-specific amplification was observed in the 5 saffron samples in presence of SAF-L40 (figure 2). A single, distinct and brightly resolved band of 412 bp was obtained in DNA isolated from the safflower varieties and no non-specific amplification was observed in the saffron samples in presence of SAF-L4. Two distinct and brightly resolved bands of 589 and 300 bp were obtained in DNA isolated from the safflower varieties and no non-specific amplification was observed in the saffron samples in presence of SAF-L70 (figure 3).

Reduction of the annealing temperatures did not generate any fragment other than the SCAR bands, confirming the specificity of the SCAR primers for all the safflower varieties. The SCAR primers were used to distinguish safflower frauds in commercial saffron, even in low level of 1%, of safflower in saffron (figure 4).

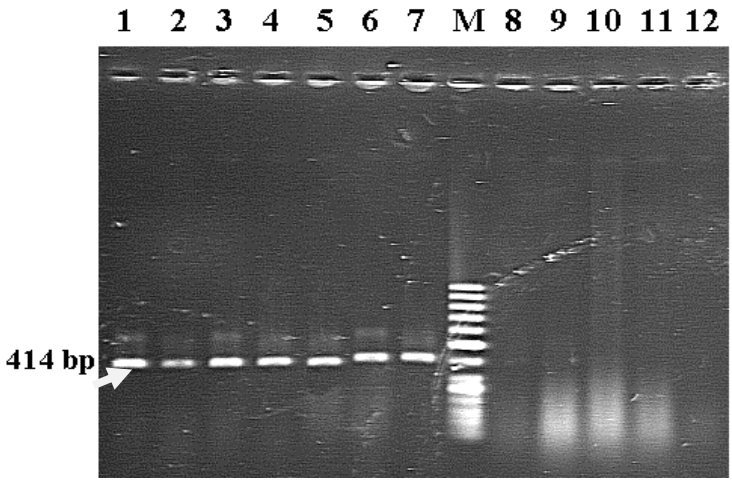

Figure 2. PCR amplification of safflower varieties using SAF-L40 on 1.2% agarose gel (M: Size marker (100 bp), lanes 1-7: safflower varieties (1: IL-111, 2: 2819, 3: 279, 4:K.W.3, 5: K.W.6, 6: K.W.16, 7: 295). Lanes 8-12: Saffron samples (8: Ghaen, 9: Gonabad, 10: Barakuh Gonabad, 11: Torbat Heidariieh, 12: Science and Technology Park).

Discussion

Identification of frauds and species is important for quality control of foods. There are examples of successful identification of species and even varieties in raw and processed materials such as textiles, seafood and plant products (Schubbert et al., 2008; Chapela et al., 2003). Molecular methods are suitable systems for tracing based on impurity in products, identification through DNA analysis. In fact DNA is unchanged and detectable in every cell, resistant to heat treatments and allows species identification (Perez and Garcia-Vazquez, 2004).

The samples which are used as fraud material are normally similar to the natural ones in morphology, and it makes their identification too difficult (Park et al., 2006). This problem can be solved by using chemical and molecular techniques specially the RAPD/SCAR method (Hernandez et al., 1999). In this study, we developed RAPD-SCAR marker for identification of safflower impurities (1%) in Saffron. In our RAPD analysis, non significant genetic polymorphism was observed among the safflower varieties and saffron samples. We selected two monomorphic bands SAF-L473 and SAF-L717 in safflower varieties for SCAR marker development. In SCAR, pairs of 20-25 bp specific primers can be used to amplify the characterized regions from genomic DNA under stringent conditions, which makes these markers more specific.

These results confirm the application of the designed primers as a qualitative diagnostic tool for identification of safflower impurities in saffron. However, for quantitative analysis of safflower content in the commercial saffron samples, advanced techniques such as real time PCR could be examined. Nevertheless, there is a pool of materials that can be used as adulterant for saffron. The adulterant may be phyllogenetically close or distinct from saffron and we are in process of developing primers for identification of such frequently used adulterants. In previous methods such as biochemical procedures, we had much impurities and it was also time consuming. So, this method enjoys from advantages of detection of impurities as low as 1% which is far from expectations in biochemical detection methods. This method is also used in frauds detection in some pharmaceutical plants such as *Phyllanthus emblica* and other economic plants such as *Bambusa* species, where the quality and quantity of paper pulp is greatly influenced by species (Das et al., 2004).

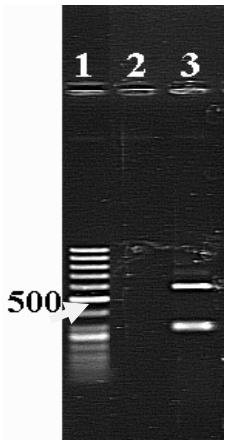

Figure 3. PCR amplification of safflower and saffron using SAF-L70 on 1.2% agarose gel (lane1: size marker (100 bp), lane2- PCR of saffron DNA (all of saffron samples) by SAF-70 primers, lane3: PCR with DNA mixture of safflower varieties by SAF-70 primers.

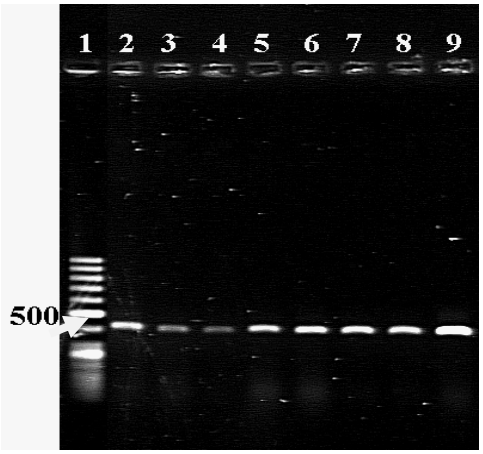

Figure 4. PCR amplification of genomic DNA extracted from mixed plant material of safflower in saffron using SAF-L4 on 1.2% agarose gel , (The numbers represent percentage of safflower in saffron samples as follows: 1: Size marker (50 bp), 2: 1% , lanes 3 and 4: 1.5%, lanes 5 and 6: 2.5%, 7: 5 % , 8:7%, 9: 10%).

Table1. The sequences of RAPD primers used in this experiment.

RAPD Primer	Primer sequence
RAP1	5′AACGACGAGCGTGAC 3′
RAP2	5′ GACAGCTTATCATCG 3′
RAP3	5′ ATGCAGGAGTCGCAT 3′
RAP4	5′AGTCATGCAACGCGC 3′
RAP5	5′GTATCACGAGGCCCT 3′
RAP6	5′GCTAGAGTAAGTAGT 3′
RAP7	5′ATGCGTCAGGCGTAG 3′
RAP8	5′TGCACTGCAGTGCAC 3′
RAP9	5′ GACTCCTGGATACCG 3′
RAP10	5′ GTAATACGACGGCCA 3′

Table 2. SCAR primers.

Primer	Sequence	Tm (ºC)
SAF-L 40 Forward	5′CCTCTCCTTTAACCCGAACAG 3′	58
SAF-L 40 Reverse	5′ATGGACTGAAGCTGGAATGAG 3′	
SAF-L 70 Forward	5′ TGAGCAGAGGAGGAGACTTG 3′	58
SAF-L 70 Reverse	5′GCCCTCAAGAAGAATACAGAGG 3′	
SAF-L 4 Forward	5′CCTCTCCTTTAACCCGAACAGCC 3′	60
SAF-L 4 Reverse	5′GGACTGAAGCTGGAATGAGAATAAC 3′	

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References

1- Amir Ghasemi T. (2001) Saffron, Red Gold of Iran, Future Press, Tehran, Iran.

2- Caetano Anolles G., Bassam B. J. and Gresshoff P. M. (1991) DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Biotechnology 9: 553-557.

3- Chapela M., Sotelo C. and Ricardo P. (2003) Molecular identification of cephalopod species by FINS and PCR-RFLP of a cytochrome b gene fragment. European Food Research and Technology 217: 524-529.

4- Cuko L., Mitsopoulou T. and Tsimidou M. Z. (2003) A rapid procedure for the evaluation of Saffron colouring strength using tristimulus colorimetry". Acta Horticulturae 650: 22-25.

5- Das M., Bhattacharya S. and Pal A. (2004) Generation and characterization of SCARs by cloning and sequencing of RAPD products: a strategy for species–specific marker development in bamboo. Annals of Botany 95: 835-841.

6- Devaiah K. M. and Venkatasubramanian P. (2008) Genetic characterization and authentication of Embelia ribes using RAPD-PCR and SCAR marker. Planta Medica 74: 194-6.

7- Dnyaneshwar W., Preeti C., Kalpana J. and Bhushan P. (2006) Development and application of RAPD-SCAR marker for identification of *Phyllanthus emblica* LINN. Biological and Pharmaceutical Bulletin 29: 2313-2316.

8- Dovwri S., Donal M. and Lee D. (2006) Non-concordance between genetic profiles of olive oil and fruit: a cautionary note to the use of DNA markers for provenance testing. Journal of Agricultural and Food Chemistry 54: 9221-9226.

9- Fernandez J. A. (2004) Biology, biotechnology and biomedicine of saffron". Recent Research of Development in Plant Science 2: 127-159.

10- Fossati T., Zapelli I., Bisoffi S., Micheletti A., Vietto L., Sala F. and Castiglione S. (2005) Genetic relationships and clonal identity in a collection of commercially relevant popular cultivars assessed by AFLP and SSR. Tree Genetics and Genomes 1: 11-20.

11- Haghighi B., Feizy J. and Hemati Kakhki A. (2007) LC Determination of Adulterated Saffron Prepared by Adding Styles Colored with Some Natural Colorants. Chromatographia 66: 325–332.

12- Hernandez P., Martin A. and Dorado G. (1999) Development of SCARs by direct sequencing of RAPD products: a practical tool for the introgression and marker-assisted selection of wheat. Molecular Breeding 5: 245-253.

13- Hernandez P., Rosa R. D. L., Rallo L., Martin A. and Dorado G. (2001) First evidence of retrotransposon like element in olive (*Olea europea*): Implications in plant variety identification by SCAR marker development. Theoretical and Applied Genetics 102: 1082-1087.

14- Kafi M., Rashed Mohasel M. H., Koocheki A. R., Molafilabi A. (2003) Saffron (*Crocus sativus*): Production and Processing. Ferdowsi University of Mashhad Press. 276p.

15- Konieczny A. and Ausubel F. M. (1993) A procedure for mapping Arabidopsis mutations using co-dominant ecotypespecific PCR-based markers. The Plant Journal 4: 403 - 410.

16- Lage M. and Cantrell C. L. (2009) Quantification of saffron (*Crocus sativus* L.) metabolites crocins, picrocrocin and safranal for quality determination of the spice grown under different environmental Moroccan conditions. Scientia Horticulturae 121 :366–373.

17- Lesur C., Becher A., Wolff K., Weising K., Steinmetz K., Peltier D. and Boury S. (2001) DNA Fingerprinters for Pelargonium cultivar identification. Acta Horticulturae 546: 325-330.

18- Lozano P., Castellar M. J., Simancas M. J. and Iborra J. L. (1999) Quantitative high-performance liquid chromatographic method to analyze commercial

saffron (*Crocus sativus* L.) products. Journal of Chromatography 830: 477–483.

19- Mafra I., Ferreira I. M. P. L. V. O. and Oliveira M. B. P. P. (2008) Food authentication by PCR-based methods. European Food Research and Technology 227: 649-665.

20- Maggi L., Sanchez A. M., Carmona M., Kanakis C. D., Anastasaki E., Tarantilis P. A. Polissiou M. G. and Alonso G. L. (2011) Rapid determination of safranal in the quality control of saffron spice (*Crocus sativus* L.) Food Chemistry. In Press.

21- Pardo J., Fernandez J. A. and Gomez L. G. (2003) Development of molecular markers for origin determination in Saffron". Acta Horticulturae 650: 22-25.

22- Park M., Kim M., In J. and Yang D. (2006) Molecular identification of Korean ginseng by amplification refractory mutation system-PCR. Food Research International 39: 568-574.

23- Perez J. and Garcia-Vazquez E. (2004) Genetic identification of nine hake species for detection of commercial fraud. Journal of Food Protection 67: 2792-2796.

24- Prakash S. and Van Staden J. (2008) Genetic variability and species identification within Encephalartos using Random Amplified Polymorphic DNA (RAPD) markers. South African Journal of Botany 74: 735-739.

25- Reid L. M., O Donnell C. P. and Downey G. (2006) Recent technological advances for the determination of food authenticity. Trends in Food Science and Technology 17: 344-353.

26- Saghai-Maroof M. A., Soliman K. M., Jorgensen R. A. and Allard R.W. (1984) Ribosomal DNA sepacer-length polymorphism in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proceedings of the National Academy of Sciences of the United States of America 81: 8014-8019.

27- Schubbert R., Hell W., Brendel T., Rittler S., Schneider S. and Klöpper K. (2008) Food forensics: Analysis of food, raw and processed materials with molecular biological methods. Forensic Science International: Genetics Supplement Series 1: 616-619.

28- Trade Promotion Organization of Iran (2009) A report of Saffron. WWW.tpo.ir.

29- Vidal J. R., Delavault P., Coarer M. and Defontaine A. (2000) Design of grapevine (*Vitis vinifera* L.) cultivar-specific SCAR primers for PCR fingerprinting. Theoretical and Applied Genetics 101:1194–1201.

30- Vidal A. O., Schnerr H., Rojmyr M., Lysholm F. and Knight A. (2007) Quantitative identification of plant genera in food products using PCR and pyrosequencing technology. Food Control 18: 921-927.

31- Tsimidou M. and Tsatsaroni E. (1993) Stability of saffron pigments in aqueous extracts. Journal of Food Science 58: 1073–1075.

32- Welsh J. and McClelland M. (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18: 7213-7218.

33- Williams J. G. K., Kubelik A. R., Livak K. J., Rafalski J. A. and Tingey S. V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18: 6531-6535.

34- Zalacain A., Ordoudi S. A., Blazquez I., Diaz-Plaza E. M., Carmona M., Tsimidou M. Z. and Alonso G. L. (2005) Screening method for the detection of artificial colours in saffron using derivative UV-Vis spectrometry. Food Additive and Contaminants 22: 607-615.

35- Zhang J., Wang H. and Cai Z. (2006) The application of DGGE and AFLP-derived SCAR for discrimination between Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Food control 18:672-676.

36- Zietkiewicz E., Rafalski A. and Labuda D. (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20: 176-183.