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# Molecular and biochemical evaluation related to fragrance in some Iranian rice varieties

**Seyed Hossein Hashemi[1], Asa Ebrahimi[1*], Amin Azadi[2]**

*[1]Department of Biotechnology and Plant Breeding, Islamic Azad University, Science and Research Branch, P. O. Box: 147789-3855, Tehran, Iran.*
*[2]Department of Plant Breeding, Yadegare-e-Imam Komeini (RAH) Shahre Rey Branch, Islamic Azad University, Tehran, Iran.*
*[*]Corresponding author, Email: asaebrahimi@srbiau.ac.ir. Tel: +98-21-44865154. Fax: +98-21-44865158.*

Received: 17 Dec 2018; Accepted: 11 Nov 2019.
DOI: 10.30479/ijgpb.2019.9781.1214

## Abstract

Rice fragrance is one of the most important and determining factors in rice quality. Aromatic rice is a special group considered to be of the best quality. It is important to know the physiological behavior and genetic source of aromatic rice in order to improve breeding programs. In this research, thirteen rice cultivars were used for molecular and biochemical evaluations. The research population included three aromatic cultivars and ten cultivars from Khuzestan province in Iran. Thirteen SSR and four MAS markers related to grain aroma were used for genotyping the rice cultivars. The highest PIC for SSR markers was 0.75. Cluster analysis using the UPGMA method classified all cultivars into three groups. The results of MAS for EAP and INSP primers showed a molecular band of 355 bp in the non-aromatic rice cultivars. In this study, four non-aromatic cultivars (Hamar (Sorkheh), Danial, Garde Ramhormoz, and Hoveizeh) were detected. The results of IFAP and ESP primers further revealed a band of 257 bp, identified in Tarom, Domsiah, Basmati, Anbarbo Najafi, Anbarbo Red, Anbarbo Yellow, Kadoos, Champa, and Shafagh, all aromatic cultivars. Moreover, the volatile components of rice seed samples were extracted and identified using the sensitive and efficient solid phase extraction method, GC-MS. Eight compounds (aldehyde, pentane, hexanal, heptane, tetradecane, ketone, acetic acid, and 2-acetyl-1-pyrroline) were identified from the studied cultivars as the most important chemical compositions of aroma in rice. The 2-AP compound was recognized in Tarom, Domsiah, and Anbarbo Najafi cultivars. Finally, four MAS primers identified all aromatic rice cultivars as well as thirteen SSR markers related to rice fragrance.

***Key words:*** GC-MS, MAS marker, Rice, SSR, 2-acetyl-1-pyrroline.

## ABBREVIATIONS

SNPs (Single nucleotide polymorphisms), MAS (Marker assisted selection), QTL (Quantitative trait loci), GC-MS (Gas chromatography–mass spectrometry), SPME (Solid-phase microextraction), EAP (External antisense primer), ESP (External sense primer), IFAP (Internal fragrant primer), INSP (Internal non fragrant sense primer), 2-AP (2-acetyl-1-pyrroline), BADH2 (Betaine Aldehyde Dehydrogenase enzyme).

## INTERDICTION

Rice (*Oryza sativa*) is an important crop that feeds more than half of the world's population. It also plays an important role in food security (Singh *et al*., 2010). Aroma and quality of taste are important criteria for consumers and are important in the market (Perez and Juliano, 1988). Aroma is not only one of the most important qualities for determining the quality of rice, but aromatic rice has a better dietary value and more amino acids, lysine, phenylalanine,

leucine, and methionine in comparison with non-aromatic cultivars (Sun *et al*., 2008). Genetic studies have shown that a recessive gene *(fgr)* is associated with chromosome 8 in aromatic rice (Ahn *et al*., 1992; Bradbury *et al*., 2005; Lang & Buu, 2008). The gene *fgr* shows a significant polymorphism among aromatic rice cultivars compared to non-aromatic cultivars. A similar situation with respect to the betaine aldehyde dehydrogenase 2 (*BADH2)* gene and its polymorphism has been recognized among aromatic and non- aromatic cultivars (Bradbury *et al*., 2005). Mutation of the *BADH2* gene has been identified as the locus responsible for the aroma, found in Basmati and Jasmine rice. This gene is an incomplete allele that codes for *BADH*. The 8 nucleotides deletion (GATTAGGC) and the three SNPs in exon 7 of Jasmin and Basmati rice produce an early end-codon which lacks the production of the betaine aldehyde dehydrogenase enzyme (*BADH2*) synthesis (2-AP) in aromatic rice, while non-aromatic cultivars completely inhibit synthesis (2-AP) (Bourgis *et al*., 2008).

The evaluation of the genetic variability of landrace accessions can provide the basic information necessary to help properly conserve these genetic resources. It will also help breeding programs to plan crosses to incorporate this variability into the genetic background of elite rice germplasms, which, in turn, will generate new rice cultivars. Microsatellites, or SSRs, are among the most widely-used DNA markers for various purposes, including diversity, genome mapping, and varietal identification (Nagaraju *et al*., 2002). SSRs are able to identify the nature of the locus (homozygous or heterozygous condition) and have the advantage of being inexpensive, simple, rapid, and only requiring a small amount of DNA. They may also be useful for the rapid screening of rice germplasm. Simple sequence repeat is an important tool for genetic variation identification of germplasms (Powell *et al*., 1996; Ma *et al*., 2011). Several researchers have presented specific SSR markers related to rice fragrance, such as RM223, RM342A, and RM515 which are linked to the FGR aroma gene (Kibria *et al.,* 2009). The SSR marker RM223 showed the highest polymorphism (66.67%) between towels genotypes and their parents (Kibria *et al*., 2009). Temnykh *et al*. (2000) presented four SSR markers (RM223, RM210, RM342A, RM152) mapped on chromosome 8 linked to the region controlling aroma. One of the most important compounds for aromatic rice is the combination of 2-AP, which cannot easily be detected due to its high sensitivity and volatility (Grimm *et al*., 2001). More than 100 volatile components have been identified in rice aroma, including hydrocarbons, alcohols, aldehydes, ketones, acids, esters, phenols, and other compounds in cooked rice (Lang and Buu, 2008). The RG28 marker of RFLP with a 4.5-cM distance shows complete linkage with the 2-AP-producing gene (Lorieux *et al*., 1996). The results of the quantitative trait loci (QTL*)* analysis of the chemical compounds of aromatic rice showed that chromosomes 1, 4, 6, 11, and 12 in aromatic rice control the locus for volatile compounds (Fahliani *et al*., 2013). Solid phase micro-extraction (SPME) has emerged as a rapid and efficient tool for the extraction and quantification of aroma compounds (Stashenko and Martínez, 2007). It is a rapid, simple, versatile, and solvent-free technique and has integrated sampling, extraction, concentration, and sample introduction of volatile compounds into gas chromatography (GC) in a single step, resulting in high sample throughput (Pico *et al.,* 2007; Soria *et al.,* 2009). Furthermore, it is useful in analyzing less stable compounds (such as 2AP) due to the immediate transfer of compounds into the analytical instrument (Eisert and Pawliszyn, 1997). Qualitative analysis by solid phase microextraction (SPME)-based methods of 2AP in rice have been reported by several researchers (Grimm *et al.,* 2001; Wongpornchai *et al.,* 2003; Laguerre *et al.,* 2007). The objective of the current study was to determine the efficiency of marker-assisted selection (MAS) and thirteen SSR markers related to rice fragrance for differentiating aromatic rice from non-aromatic rice and identifying the volatile compounds in some aromatic rice varieties grown in southern Iran.

## MATERIALS AND METHODS

### Plant materials

In this study, 13 various rice cultivars were used, 10 of which were from southern parts of Iran (Khuzestan province) and two were from local cultivars in northern Iran as a positive control; one cultivar was an international Basmati variety (Table 1).

### Evaluation of volatile compounds in aromatic rice

In this experiment, solid-phase micro-extraction (SPME) chromatography was used to identify the compounds in aromatic rice. Samples (1.5 mg) were added into a 10-mL glass microtube along with 200 µl sterilized water, and the mixture was incubated for 10 minutes at room temperature. To extract the headspace, the microtubes containing rice and sterilized water were incubated in a Ben-Mari at 85 °C for 30 min. To absorb the material in the headspace, a super elastic SPME metal fiber (Supelco, USA) was used. To absorb the headspace, the SPME fiber was kept in a glass microtubule for 15 minutes before

**Table 1.** Names and sources of rice cultivars used in this study.

| No. | Cultivar name | Source |
|---|---|---|
| 1 | Tarom | GilanRice Research Institute of Iran - Deputy of Mazandaran |
| 2 | Domsiah | Rice Research Institute of Iran - Deputy of Mazandaran |
| 3 | Basmati | Hindustān (in Iranian market) |
| 4 | Anbarbo Najafi | Agricultural Research Education And Extention Organization of Iran - Khuzestan Province |
| 5 | Anbarbo Red | Agricultural Research Education And Extention Organization of Iran - Khuzestan Province |
| 6 | Anbarbo Yellow | Agricultural Research Education And Extention Organization of Iran - Khuzestan Province |
| 7 | Hamar (Sorkheh) | Agricultural Research Education And Extention Organization of Iran - Khuzestan Province |
| 8 | Danial | Agricultural Research Education And Extention Organization of Iran - Khuzestan Province |
| 9 | Kadoos | Agricultural Research Education And Extention Organization of Iran - Khuzestan Province |
| 10 | Garde Ramhormoz | Agricultural Research Education And Extention Organization of Iran - Khuzestan Province |
| 11 | Hoveizeh | Agricultural Research Education And Extention Organization of Iran - Khuzestan Province |
| 12 | Champa | Agricultural Research Education And Extention Organization of Iran - Khuzestan Province |
| 13 | Shafagh | Agricultural Research Education And Extention Organization of Iran - Khuzestan Province |

being injected into the HP chromatography apparatus with mass detection. Rice seed samples were used to isolate and identify volatile compounds. Depletion was performed for 5 minutes at the injection site at 270 °C. The column temperature program started with 3 min at 50 °C and then reached 250 °C at a speed of 10 °C/min. The HP-1 capillary column was used with helium as the carrier gas at a mass of 40 cm/s. The entire GC cycle was performed in 30 min with a cooling period of 5 min (Grimm *et al*., 2001). The GC-MS system was used to study the mass spectra and compare them with standard mass spectra stored in the Willey257 software library.

**Genomic DNA extraction**

DNA was extracted from the endosperm of rice seed using the modified CTAB method (Huabing *et al.,* 2016). The quality of the extracted DNA was determined by applying it on a 0.8% agarose gel and was quantitated through a spectrophotometer.

**Polymerase Chain Reaction (PCR)**

The reaction mixture (25 µl) contained each primer at a concentration of 0.5 µM, each deoxynucleoside triphosphate at a concentration of 200 µM, 2.5 U of Taq DNA polymerase, 1.5 mM MgCl2, 20 ng template DNA, and 2.5 µl 10×PCR buffer. Amplification of the DNA was performed in a thermocycler in the following manner: initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 95 °C for 45 seconds, primer annealing between 55-59 °C for 45 seconds (Table 2 and 3), and extension at 72 °C for 45 seconds, with a final extension at 72 °C for 4 min for MAS and SSR markers. The PCR products were loaded on 1% agarose gel using 1×TBE buffer containing 10 mg/ml ethidium bromide and were visualized under UV light. The gel was photographed using the UV gel documentation system.

**Selection of molecular markers**

Thirteen microsatellite markers (Table 2) were used on 13 local rice varieties selected by referring to www.gramene.org regarding coverage of all 12 rice chromosomes.

The MAS markers were capable of detecting pure aromatic homozygous, heterozygous, and non-aromatic homozygous cultivars (Table 3). These markers are characterized by three different molecular weight bands that initiate EAP and ESP with a molecular weight (577-585 bp) and act as an internal control. The presence of these bands indicates the proper functioning of the PCR. The EAP and INSP with a molecular weight of 355 bp was used to identify non-aromatic homozygous cultivars, but the IFAP and ESP with a molecular weight of 257 bp was used to identify homozygous aromatic cultivars (Bradbury *et al*., 2005) (Figure 1).

**Table 2.** SSR markers related to rice aroma used for genotyping of 13 rice varieties.

| Locus | Chromosome | Repeat motif | Annealing (°C) | | Sequence of primers |
|---|---|---|---|---|---|
| RM30 | 6 | (AG)9A(GA)12 | 55 | F | GGTTAGGCATCGTCACGG |
| | | | | R | TCACCTCACCACACGACACG |
| RM180 | 7 | (ATT)10 | 55 | F | CTACATCGGCTTAGGTGTAGCAACACG |
| | | | | R | ACTTGCTCTACTTGTGGTGAGGGACTG |
| RM182 | 7 | (AT)16 | 59 | F | TGGGATGCAGAGTGCAGTTGGC |
| | | | | R | CGCAGGCACGGTGCCTTGTAAG |
| RM190 | 6 | (CT)11 | 55 | F | CTTTGTCTATCTCAAGACAC |
| | | | | R | TTGCAGATGTTCTTCCTGATG |
| RM207 | 2 | (CT)25 | 55 | F | CCATTCGTGAGAAGATCTGA |
| | | | | R | CACCTCATCCTCGTAACGCC |
| RM223 | 8 | (CT)25 | 55 | F | GAGTGAGCTTGGGCTGAAAC |
| | | | | R | GAAGGCAAGTCTTGGCACTG |
| RM243 | 1 | (CT)18 | 55 | F | GATCTGCAGACTGCAGTTGC |
| | | | | R | AGCTGCAACGATGTTGTCC |
| RM247 | 12 | (CT)16 | 55 | F | TAGTGCCGATCGATGTAACG |
| | | | | R | CATATGGTTTTGACAAAGCG |
| RM310 | 8 | (GT)19 | 55 | F | CCAAAACATTTAAAATATCATG |
| | | | | R | GCTTGTTGGTCATTACCATTC |
| RM341 | 2 | (CTT)20 | 55 | F | CAAGAAACCTCAATCCGAGC |
| | | | | R | CTCCTCCCGATCCCAATC |
| RM515 | 8 | (GA)11 | 55 | F | TAGGACGACCAAAGGGTGAG |
| | | | | R | TGGCCTGCTCTCTCTCTCTC |
| RM577 | 1 | (TA)9(CA)8 | 55 | F | GCTTTCCCTCTAACCCCTCT |
| | | | | R | GGATGTACCGCTGACATGAA |
| RM28102 | 12 | (TATC)10 | 55 | F | CACTAATTCTTCGGCTCCACTTTAGG |
| | | | | R | GTGGAAGCTCCGAGAAAGTGC |

F: Forward, R:Reverse.

**Table 3.** Specific primers used for rice fragrance.

| Markers | Chromosome | Annealing (°C) | Forward primer | Reverse primer | Reference |
|---|---|---|---|---|---|
| ESP IFAP | 8 | 58 | TTGTTTGGAGCTTGCTGATG | CATAGGAGCAGCTGAAATATATACC | Bradbury *et al.*, (2005) |
| ESP EAP | 8 | 58 | CTGGTAAAAAGATTATGGCTTCA | CATAGGAGCAGCTGAAATATATACC | |
| INSP EAP | 8 | 58 | CTGGTAAAAAGATTATGGCTTCA | AGTGCTTTACAAAGTCCCGC | |

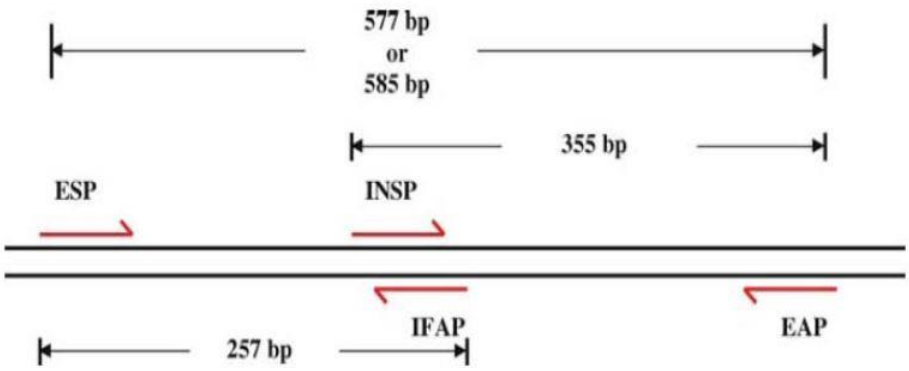


**Figure 1.** Relative positions of PCR primers “IFAP-ESP” and “EAP-INSP” used for rice fragrance.

**SSR data analysis**

The bands representing particular alleles at microsatellite loci were scored manually.

The average number of observed alleles, the effective number of alleles, and the genetic diversity based on the Shannon index were calculated using POPGENE. Ver 1.32.

Polymorphism information content (PIC) was calculated based on the Botstein *et al*. (1980) formula using Power Marker version 3.25:

Cluster analysis was carried out based on simple matching (SM) coefficients, the UPGMA algorithm and NTSYS Ver.2.02 software.

## RESULTS

**Marker Results (SSR)**

In this study, 13 SSR markers were evaluated on rice samples (Table 4), and 42 polymorphic bands (alleles) were identified. The size of alleles ranged from 105 to 500 bp (Figure 2). RM223 marker revealed the maximum number of alleles (6). RM 28102 marker showed the minimum number of alleles (1). An overall average of 3.23 alleles was determined. The most effective allele was the RM223 marker with 4.56, and the less effective marker was RM28102 with 1.35. In overall, an average of 2.35 effective alleles was determined.

The Shannon index was used as a criterion for estimating intra-population diversity. The highest Shannon index was 1.63, found in RM223 marker, and the lowest index was 0 for RM28102 marker. Overall, the average calculated Shannon index was 0.88. The highest gene diversity (Nei) was obtained for RM223 marker (0.78). An average gene diversity of 0.50 was obtained. The first and most important indicator for evaluating the usefulness of a marker is the calculation of its

**Table 4.** Characteristics of SSR markers in all the studied genotypes.

| Locus | Allele size (bp) | Observed alleles | Effective alleles | Shannon index (I) | Genetic diversity (Nei) | PIC* |
|---|---|---|---|---|---|---|
| RM 30 | 105-140 | 3 | 2.77 | 1.05 | 0.63 | 0.56 |
| RM 180 | 123-243 | 3 | 1.75 | 0.76 | 0.42 | 0.38 |
| RM 182 | 323-500 | 5 | 2.64 | 1.16 | 0.26 | 0.55 |
| RM 190 | 120-154 | 2 | 1.98 | 0.69 | 0.49 | 0.37 |
| RM 207 | 154-211 | 3 | 1.89 | 0.83 | 0.47 | 0.42 |
| RM 223 | 117-295 | 6 | 4.56 | 1.63 | 0.78 | 0.75 |
| RM 243 | 107-145 | 3 | 2.08 | 0.85 | 0.52 | 0.44 |
| RM 247 | 163-264 | 4 | 3.21 | 1.26 | 0.68 | 0.63 |
| RM 310 | 139-200 | 3 | 2.77 | 1.05 | 0.63 | 0.56 |
| RM 341 | 145-448 | 4 | 1.86 | 0.84 | 0.46 | 0.41 |
| RM 515 | 263-300 | 2 | 1.35 | 0.42 | 0.26 | 0.22 |
| RM 577 | 200-213 | 3 | 2.35 | 0.91 | 0.56 | 0.47 |
| RM 28102 | 212 | 1 | 1 | 0 | 0 | 0 |
| Mean | - | 3.23 | 2.35 | 0.88 | 0.50 | 0.44 |

*: Polymorphic Information Content.

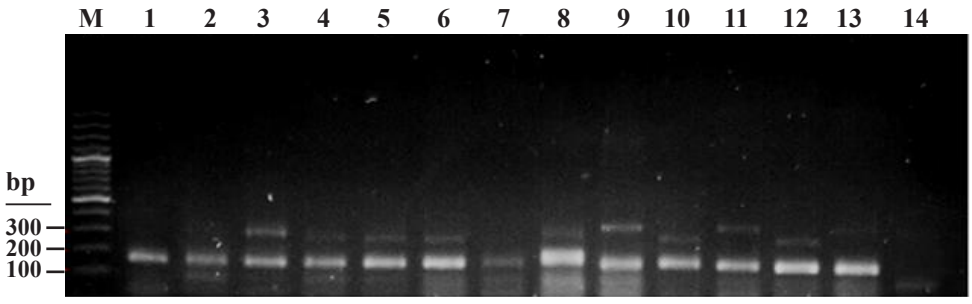


**Figure 2.** Gel electrophoresis image showing the amplification of SSR marker RM223 related to rice aroma. Lane M: ladder Gen Ruler 100 bp plus, Lane 1: Tarom, Lane 2: Domsiah, Lane 3: Basmati, Lane 4: Anbarbo Najafi, Lane 5: Anbarbo Red, Lane 6: Anbarbo Yellow, Lane 7: Hamar (Sorkheh), Lane 8: Danial, Lane 9: Kadoos, Lane 10: Garde Ramhormoz, Lane 11: Hoveizeh, Lane 12: Champa, Lane 13: Shafagh, Lane 14: DNA from control sample.

polymorphic information content (Agrama and Tuinstra, 2003). In the current study, the highest PIC and the average PIC value were obtained for the RM223 marker, which were calculated as 0.75 and 0.44, respectively.

Clustering was carried out using the UPGMA method with simple matching similarity coefficients employing the NTSYS software. Based on the merged intervals and considering the similarity coefficients, the cutting line was considered at a 70% similarity level. Items were divided into three groups (Figure 3). The first group consisted of Tarom and Domsiah cultivars with a similarity coefficient of 0.93. These figures were considered as the positive control because of their aromaticity. Given the geographical proximity to northern Iran, they are also in the same expected group. The second group consisted of the internationally known Basmati cultivar, Anbarbo Najafi, Anbarbo Red, Anbarbo Yellow, and Hamar (Sorkheh) with a similarity coefficient of 0.72. They were grouped according to the geographical location (south of Khuzestan). The aromatic cultivars of Khuzestan (Anbarbo) and Basmati were placed in the same group (group two with a similarity coefficient of 0.72). It is proposed that Anbarbo Red and Anbarbo Yellow were completely similar to the the alleles related to rice aroma. The third group consisted of Danial, Kadoos, Garde Ramhormoz, Hoveizeh, Champa, and Shafagh cultivars with a similarity coefficient of 0.73. They are located at the geographical proximity in the north, northeast, and northwest regions of Khuzestan.

**Marker Results (MAS)**

The MAS markers in this research were used to identify aromatic and non-aromatic cultivars. This marker effectively distinguished pure aromatic cultivars from non-aromatic and heterozygous ones. The primers "IFAP+ESP", "EAP+INSP", and "EAP+ESP" produced three cultivars of bands with molecular weights of 580, 355, and 257 bp, respectively (Figure 4). The paired primers "EAP+ESP" with a 580-bp band was estimated as the initiator act and internal control. The presence of this band indicates the proper functioning of the PCR, observed for all samples tested with a 580-bp band.

The second band obtained with a molecular weight of 355 bp was derived from the EAP and INSP primer pair which represented non-aromatic homozygous cultivars. In the present research, 4 non-aromatic rice cultivars were detected (Hamar (Sorkheh), Danial, Garde Ramhormoz, and Hoveizeh).

The third pair of primers "IFAP–ESP" presents a band with a molecular size of 257 bp, which determined aromatic homozygous cultivars. In this study, 9 aromatic landraces were detected (Tarom, Domsiah, Basmati, Anbarbo Najafi, Anbarbo Red, Anbarbo Yellow, Kadoos, Champa, and Shafagh). Among the aromatic cultivars identified through the MAS marker, Tarom, Domsiah, and Basmati cultivars were considered as the positive control. This indicates the proper separation of rice varieties by IFAP and ESP.

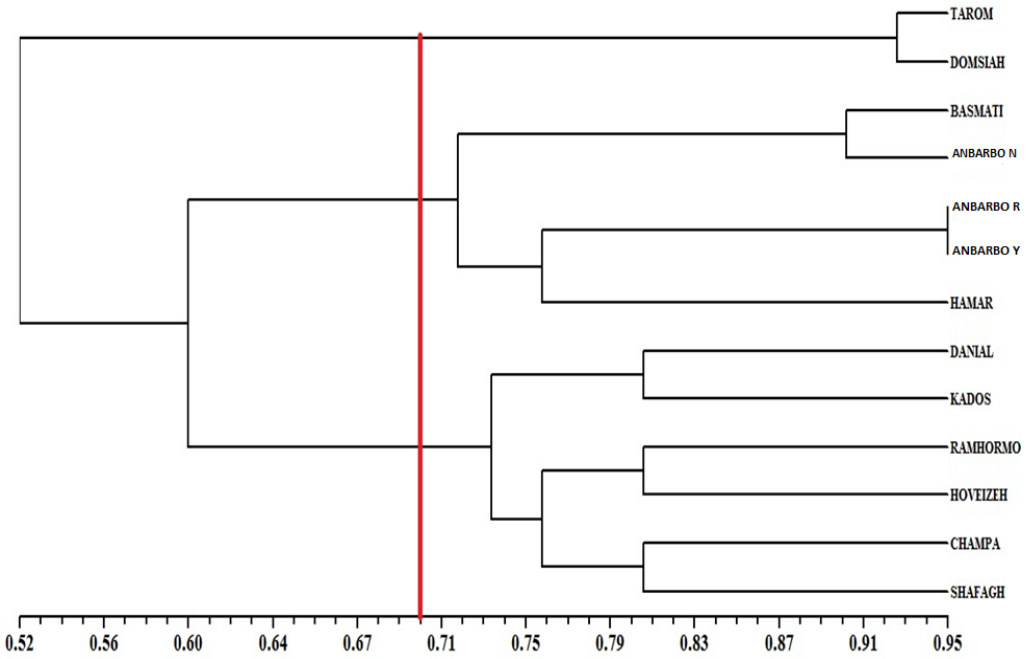


**Figure 3.** Dendrogram derived from UPGMA cluster analysis, showing genetic diversity among 13 rice varieties.

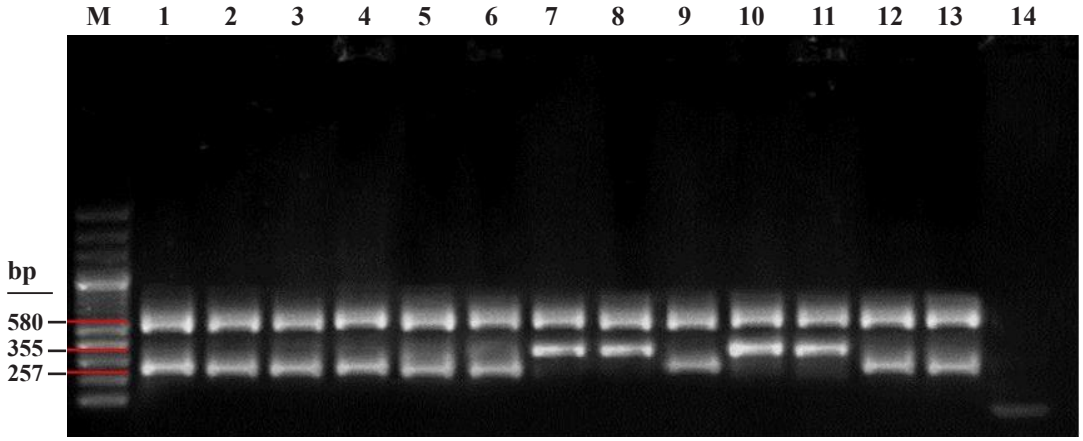


**Figure 4.** Gel electrophoresis image showing the amplification of MAS markers related to rice aroma. Lane M: ladder Gen Ruler 100 bp plus, Lane 1: Tarom, Lane 2: Domsiah, Lane 3: Basmati, Lane 4: Anbarbo Najafi, Lane 5: Anbarbo Red, Lane 6: Anbarbo Yellow, Lane 7: Hamar (Sorkheh), Lane 8: Danial, Lane 9: Kadoos, Lane 10: Garde Ramhormoz, Lane 11: Hoveizeh, Lane 12: Champa, Lane 13: Shafagh, Lane 14: DNA extracted from control sample.

In conclusion, four non-aromatic and nine aromatic rice landraces were determined using three primer pairs "IFAP+ESP", "EAP+INSP", and "EAP+ESP".

**Phenotypic Results**

In the present research, SPME extraction chromatography was used to confirm the molecular marker results and to identify important aromatic compounds in Iranian rice cultivars. In this study, 29 volatile compounds from 13 rice cultivars were identified (Table 5).

The main compounds found in aromatic rice include 2-acetyl-1-pyrroline, propanol, pentane, hexanal, heptane, tetradecane, hydrocarbons, aldehydes, ketones, esters, and phenols. These compounds existed in all aromatic cultivars. Using the analysis of these compounds, Tarom cultivar (positive control) with 11 compounds, Anbarbo Najafi cultivar with 10 compounds, Domsiah cultivar with 9 compounds, Kadoos, Champa, Shafagh cultivars with 7 compounds, Anbarbo red, Anbarbo yellow and Basmati cultivars with 6 compounds had the most aromatic compounds and were considered to be aromatic. Conversely, the Hamar (Sorkheh) and Danial cultivars with 2 compounds and the Garde Ramhormoz and Hoveizeh cultivars with 5 compounds contained the lowest number of aromatic compounds and were considered to be non-aromatic. In this experiment, 2-acetyl-1-pyrroline (2-AP), one of the most important compounds for rice aroma, was also identified in the aromatic varieties (Figure 5).

The 2-acetyl-1-pyrroline (2-AP) was detected in Tarom, Domsiah, and Anbarbo Najafi cultivars. The other cultivars did not contain this important aromatic compound (2-AP). Moreover, hexanal was detected in all studied cultivars. Cyclotrisiloxane and furan 2-penty1 compounds were also detected in all studied rice cultivars except Hamar (Sorkheh). Four compounds, thymol acetate, sesamin, eicosane, and heneicosane, were absent in the aromatic rice cultivars. Heneicosane existed only in Hamar (Sorkhe) cultivar (non-aromatic cultivar). 2-AP was extracted from the commercial fiber, using the SPME method, and with the LECO ChromaTOF software. The mass of 2-AP in the volatile compounds was shown to be 111 *m/z* (Figure 6A) by the mass spectrometry. The mass of 2-AP given by the Willey257 software library in the GC-MS system was very similar to that of 2-AP collected from the samples tested (Figure 6B).

## DISCUSSION

Huabing *et al.* (2016) extracted DNA from rice endosperm using CTAB 2% with the addition of alpha amylase. Sajib *et al*. (2017) also used 10-12 g of grain rice and 100 µl of Chelex®-100 to extract DNA from rice. Sajib *et al*. (2012) evaluated 12 rice genotypes using 9 molecular markers. They reported that the average number of observed alleles was 3.33, genetic diversity was 0.54, and PIC was 0.48. Kibria *et al*. (2009) studied 14 rice cultivars using 3 molecular markers and reported an average Shannon index value of 0.88, 3 alleles, 2.19 effective alleles, and heterozygosity of 0.11. In the present study, the gene diversity was also found to be 0.54.

Prathepha (2012) studied 94 native Thai cultivars using 7 microsatellite markers and reported an average PIC of 0.71. In the present study, the best PIC value was obtained for RM223 (0.75), and the average PIC value was 0.44.

**Table 5.** Chemical compounds detected from 13 rice samples, and resemblance amount of these compounds have been compared to GC -MS and calculated with percentages.

| No. | Compounds | Tarom | Domsiah | Basmati | Anbarbo Najafi | Anbarbo Red | Anbarbo Yellow | Hamar (Sorkheh) | Danial | Kadoos | Garde Ramhormoz | Hoveizeh | Champa | Shafagh |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Acetone | 17.92 | 13.65 | 11.31 | 15.23 | - | - | - | 12.97 | 12.72 | - | - | 9.27 | 12.38 |
| 2 | Acetaldehyde | - | 8.39 | - | 19.51 | - | - | - | - | 13.55 | - | - | - | - |
| 3 | Acetamide 2-fluoro | 38.74 | 65.19 | - | 29.7 | 53.13 | 31.84 | - | - | - | - | - | 36.49 | 24.13 |
| 4 | Asarinin | - | - | - | - | 18.73 | 12.26 | 35.67 | - | - | - | - | - | - |
| 5 | Azetidin | 11.07 | 9.43 | - | 9.83 | - | - | - | - | - | - | - | - | - |
| 6 | Benzene | 23.66 | - | 18.34 | - | 20.58 | 14.62 | - | - | 42.65 | - | - | 19.24 | 22.33 |
| 7 | Cyclopentane | - | - | - | - | - | - | - | 80.54 | - | - | - | 49.13 | 56.07 |
| 8 | Aziridine | 17.47 | 14.09 | - | 21.38 | - | - | - | - | - | - | - | - | - |
| 9 | Propenal | 51.53 | 37.27 | 45.16 | 21.45 | 36.54 | 32.84 | - | - | 19.51 | 48.83 | 62.49 | 42.19 | 39.11 |
| 10 | Ethanamine | 33.05 | - | - | 26.34 | - | - | - | - | - | - | - | - | - |
| 11 | Pentaene | 21.81 | 39.18 | - | - | - | - | - | 24.66 | 18.01 | 58.32 | 50.42 | - | - |
| 12 | Hexanal | 59.09 | 51.42 | 64.02 | 42.12 | 62.26 | 59.2 | 65.72 | 58.06 | 53.71 | 60.17 | 80 | 57.2 | 66.12 |
| 13 | Cyclotrisiloxane | 13.26 | 21.18 | 9.41 | 12.18 | 13.03 | 11.27 | - | 14.03 | 15.07 | 13.08 | 15.56 | 14.2 | 14.03 |
| 14 | Ethene | 16.33 | 12.27 | - | 13.27 | 7.19 | 5.47 | - | 3.12 | 9.61 | 12.62 | 30.55 | 9.21 | 6.37 |
| 15 | 1-Isopropenyl | 36.95 | 31.15 | - | - | - | - | - | - | - | 21.09 | 19.51 | 33.19 | 37.11 |
| 16 | 2-Acetyl-1 Pyrroline | 29.13 | 16.65 | - | 23.07 | - | - | - | - | - | - | - | - | - |
| 17 | Heptanal | 17.56 | - | 18.49 | 19.47 | 50.66 | 63.17 | 93.17 | 48.01 | - | 73.11 | 56.25 | 43.18 | 15.46 |
| 18 | Phenonl | - | - | - | - | 18.79 | 13.43 | - | - | - | 12.76 | 16.42 | - | - |
| 19 | Limonene | 35.22 | 30.83 | - | 33.05 | - | - | - | - | - | - | - | 24.53 | 29.31 |
| 20 | Sabinene | 30.07 | 21.14 | - | 26.17 | 11.51 | - | - | - | - | - | - | - | - |
| 21 | Beta-phellandrene | 28.13 | 18.32 | - | 23.49 | 14.03 | - | - | - | - | - | - | 16.37 | 18.25 |
| 22 | Furan 2-penty1 | 24.53 | 21.47 | 13.79 | 23.18 | 15.46 | 14.36 | - | 15.87 | 12.08 | 16.36 | 38.57 | 16.49 | 14.03 |
| 23 | Imidazole-2-d1 | 22.93 | - | - | 17.41 | - | - | - | - | - | - | - | - | 15.39 |
| 24 | Acetic acid | 30.71 | - | - | 23.75 | - | - | - | - | - | - | - | - | - |
| 25 | Tetra decane | 47.97 | 29.23 | 17.46 | 53.19 | 27.16 | 24.29 | - | 85.73 | 46.39 | - | - | 36.11 | 24.71 |
| 26 | Thymol acetate | - | - | - | - | - | - | - | - | - | - | 23.44 | - | - |
| 27 | Sesamin | - | - | - | - | - | - | 37.05 | - | - | - | - | - | - |
| 28 | Eicosane | - | - | - | - | - | - | 67 | - | - | - | 67.42 | - | - |
| 29 | Heneicosane | - | - | - | - | - | - | 68.43 | - | - | - | - | - | - |

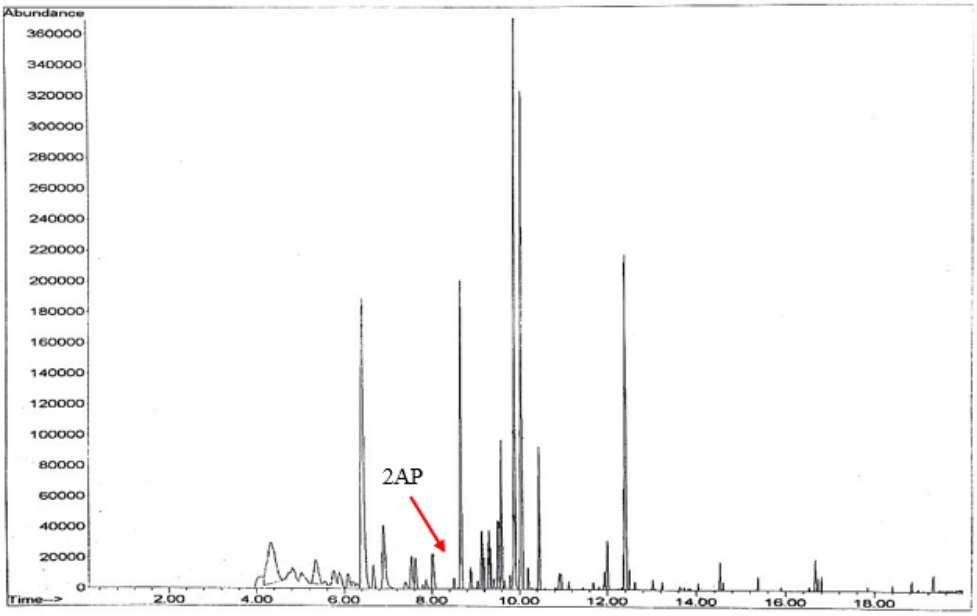


**Figure 5.** Relative rice extract compounds using SPME commercial fibers.

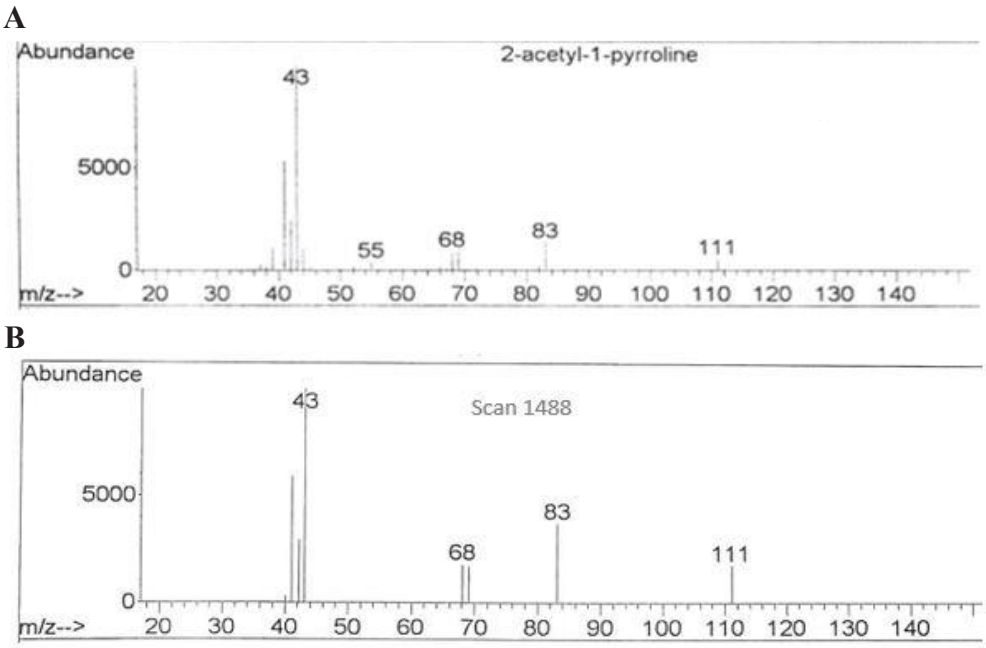


**Figure 6.** The mass spectrum 2AP derived from GC/MS.

Palanga *et al.* (2016) showed that SSR marker RM 342 had the highest PIC value and could be used to differentiate aromatic varieties from nonaromatic ones. They also reported RM 190, which was very informative on the cooking and eating parameters.

High PIC values indicate high polymorphism and existence of alleles or rare alleles in a marker position (Ribeiro Carvalho *et al*., 2004). Ashfaq *et al.* (2015) studied 75 rice cultivars gathered from the Shah Kakoo Commodity Research Institute in Pakistan. The MAS marker was used to genetically screen this rice population, and 28 aromatic homozygous cultivars were identified by primers “IFAP-ESP”. Moreover, 6 heterozygote cultivars were detected by primers “IFAP-ESP” and “EAP-INSP”. Also 41 non-aromatic homozygous cultivars were detected by primers "EAP-INSP". In another research, 168 cultivars of an F2 rice population collected from Australia were evaluated using MAS marker, and 46 aromatic homozygous rice cultivars were detected by primers “IFAP–ESP”, 80

heterozygote cultivars detected by primers "IFAP–ESP" and "EAP–INSP", and 42 non-aromatic homozygous cultivars were detected by primers "EAP –INSP" (Bradbury *et al.,* 2005).

Marker-assisted selection is usually used to detect aromatic and non-aromatic rice, although complementary biochemical research is also necessary. In this study, 4 non-aromatic Iranian rice cultivars (Hamar (Sorkheh), Danial, Garde Ramhormoz, and Hoveizeh) as well as nine Iranian aromatic rice cultivars (Tarom , Domsiah, Basmati, Anbarbo Najafi, Anbarbo Red, Anbarbo Yellow, Kadoos, Champa, and Shafagh) were successfully detected by marker-assisted selection. Complementary biochemical evaluation was also used to confirm the MAS results. GC-MS and HPLC methods were used for the determination of aroma compounds. Perhaps the limitations of the method of isolation and quantification might be responsible for the considerable delay the previous researchers experienced in detecting 2-AP molecule. The 2-AP content in milled Basmati was reported to vary from 0.06 (Buttery *et al.,* 1983) to 0.588 (Tava and Bocchi, 1999) to 0.061 ppm (Nadaf *et al.,* 2006) using steam distillation; from 0.019–0.342 ppm (Bergman *et al.,* 2000) to 0.235 ppm (Yoshihashi 2002) by solvent extraction; and from 0.26–0.38 ppm by static headspace-GC (Sriseadka *et al.,* 2006). Bergman *et al*. (2000) evaluated 15 different rice samples and detected the 2-AP in all samples using the GC-MS method. The Aromatic "Fowler Gourmet" cultivars with 999 ng, Jasmine (ITC) with 810 ng, Jasmine (Millagrosa MRR) with 598 ng, and Jasmine (Fantastic Foods) with 550 ng of 2-AP were identified. In Basmati Easy Cook (Tilda) with 19 ng and Basmati (Fantastic Foods) 68 ng of 2-AP were also detected. Therefore, it seems that the amount of 2-AP is an important factor in defining aromatic rice. In the present study, 2-AP compound was also detected using GC-MS in three Iranian rice cultivars which had been selected by MAS in the first section as aromatic rice. However, lower amounts of 2-AP were reported in black rice (Nadaf *et al.,* 2006) and in six rice flavor cultivars (Ghiasvand *et al.,* 2007). In a research, four samples from Lorestan province in the west of Iran, five aromatic rice samples from the northern provinces, and two samples of Indian rice were used. The Doroud Tarom, Doroud, and the Domsiah rice cultivars, showed maximum amounts of 2-AP and Basmati Gold Rice and Basmati had the minimum amount of 2-AP.

In the current study, four compounds (thymol acetate, sesamin, eicosane, and heneicosane) were not found in aromatic rice and could be considered as non-important compositions in Iranian rice aroma. However, other compounds such as alkanes, tetradecan, pentadecane, hexadecane, and heptidacane are some of the other volatile chemical compounds found in rice. These compounds have also been reported by other researchers (Widjaja *et al.,* 1996; Singh *et al.,* 2000). It can be concluded that the other aromatic rice cultivars detected by MAS in this study probably possess other volatile compounds as well. Furthermore, hexanal was identified in all studied cultivars. In this research acetone was also detected in 9 cultivars, including the most aromatic ones which had the 2-AP (Tarom, Domsiah, and Anbarbo Najafi). It was also detected in Tarom, Domsiah, Basmati, Anbarbo Najafi, Danial, Kadoos, Champa, and Shafagh cultivars. Pentaene was detected in Tarom, Domsiah, Danial, Kadoos, Garde Ramhormoz, and Hoveizeh cultivars. Heptanal was detected in all cultivars except Domsiah and Kadoos. In this research, the azetidine compounds from the pyrazine family with an appearance of solid crystals were detected only in Tarom, Anbarbo Najafi, and Domsiah cultivars (the most aromatic rice). The effect of rice whitening on its aroma has been evaluated, and it has been reported that the outer layers of rice has an important role in making its aroma (Bullard & Holguin, 1977).

## CONCLUSION

According to different criteria calculated in different rice groups, it can be said that there was genetic diversity in each group of cultivars and between groups. In the present study, the local rice cultivars of Khuzestan were placed in two groups (north and south of Khuzestan). Joint microsatellite markers with fragrance quality characteristics could distinguish the high-quality fragrance rice samples from the other rice samples.

In this research, the genetic control (which acts as a recessive single gene) of aroma in rice was studied and the efficiency of molecular markers was determined. These initiators distinguished pure aromatic cultivars from non-aromatic and heterozygous ones. The phenotypic test was used to identify the chemical composition of the volatile aromatic rice varieties and evaluate them with a chromatographic device, a powerful system for the rapid and easy screening of a wide range of substances, which identified the combination of 2-acetyl-1-pyrrolin as a key compound in aromatic cultivars. Among cultivars Khuzestan, Anbarbo Najafi, Domsiah, Kadoos, Champa, Shafagh, Red Anbarbo, Yellow Anbarbo, and Basmati had the most aromatic compounds and are considered to be aromatic cultivars. Hamar (Sorkheh), Danial, Garde

Ramhormoz, and Hoveizeh had the lowest number of aromatic compounds and are considered to be non-aromatic cultivars. These results were confirmed by the MAS markers in this study.

## ACKNOWLEDGEMENTS

I would like to express my sincere thankfulness to Mr. Assareh, from science and research branch laboratory for guidance and support in my project.

## REFERENCES

Agrama H. A., and Tuinstra M. R. (2003). Phylogenetic diversity and relationships among sorghum accessions using SSRs and RAPDs. *African Journal of Biotechnology*, 2: 334–340.

Ahn S. N., Bollich C. N., and Tanksley S. D. (1992). RFLP tagging of a gene for aroma in rice. *Theoretical and Applied Genetics*, 84: 825–828.

Ashfaq M., Farooq H. U., Sabar M., Ali A., and Ali M. (2015). Molecular screening of aroma genes in indigenous and exotic rice germplasm. https://www.researchgate.net/publication,311575183.

Bergman C. J., Delgado J. T., Bryant R., Grimm C., Cadwallader K. R., and Webb B. D. (2000). Rapid gas chromatographic technique for quantifying 2-acetyl-l-pyrroline and hexanal in rice (*Oryza sativa* L.). *Cereal Chemistry*, 77: 454–458.

Botstein D., white R. L., Skolnick M., and Davis R. W. ( 1980). Construction of a genetic linkage map in man using restriction fragment length polymorphism. *American Journal of Human Genetics,* 32(3): 314–331.

Bourgis F., Guyot R., Gherbi H., Tailliez E., Amabile I., Salse J., Lorieux M., Delseny M., and Ghesquière A. (2008). Characterization of the major fragance gene from an aromatic japonica rice and analysis of its diversity in Asian cultivated rice. *Theoretical and Applied Genetics*, 117: 353–368.

Bradbury L. M. T., Henry R. J., Jin Q. S., Reinke R. F., and Waters D. L. E. (2005). A perfect marker for fragrance genotyping in rice. *Molecular Breeding*, 16: 279–283.

Bullard R.W., and Holguin G. (1977).Volatile components of unprocessed rice (*Oryza sativa*). *Journal of Agricultural and Food Chemistry*, 25(1): 99–103. DOI: 10.1021/jf60209a050.

Buttery R. G., Ling L. C., Juliano B. O., and Turnbaugh J. G. (1983). Cooked rice aroma and 2-acetyl-1-pyrroline. *Journal of Agricultural and Food Chemistry,* 31: 823–826.

Buttery R., Turnbaugh J., and Ling L. (1988). Contributions of volatiles to rice aroma. *Journal of Agricultural and Food Chemistry,* 36: 1006–1009.

Eisert R., and Pawliszyn J. (1997). New trends in solid-phase microextraction. *Critical Reviews in Analytical Chemistry*, 27(2): 103–135.

Fahliani A. R., Khodambashi M., and Ghiasvand A. R. (2013). Identification of rice aromatic compounds by solid phase micro-extraction method in GC-MS and mapping their controlling QTLs using microsatellite marker. *Journal of Agricultural Biotechnology,* 5(3): 19–31.

Ghiasvand A. R., Setkova L., and Pawliszyn J. (2007). Determination of flavour profile in Iranian fragrant rice samples using cold-fibre spme–gc–tof–ms. *Flavour and Fragrance Journal,* 22(5): 377–391. Doi: 10.1002/ffj.1809.

Grimm C. C., Bergman C., Delgado J. T., and Bryant R. (2001). Screening for 2-acetyl-1-pyrroline in the headspace of rice using SPME/GC–MS. *Journal of Agricultural and Food Chemistry,* 49: 245–249.

Huabing L., Ya D., Chuntai W., and Xin X. (2016). A high-throughput DNA extraction method from rice seeds. *Biotechnology & Biotechnological Equipment,* 30(1): 32–35. DOI: 10.1080/13102818. 2015.1088401.

Kibria k., Nur F., Begum S. N., Islam M. M., Paul S. K., Rahman K. S., and Azam S. M. M. (2009). Molecular marker based genetic diversity analysis in aromatic rice genotypes using Ssr and Rapd markers. *International Journal of Sustainable Crop Production,* 4(1): 23–34.

Laguerre M., Mestres C., Davrieux F., Ringuet J., and Boulanger R. (2007). Rapid discrimination of scented rice by solid phase microextraction, mass spectrometry and multivariate analysis used as a mass sensor. *Journal of Agricultural and Food Chemistry*, 55: 1077–1083.

Lang N. T., and Buu B. C. (2008). Development of PCR-based markers for aroma (*fgr*) gene in rice (*Oryza sativa* L.). *Omonrice*, 16: 16–23.

Lorieux M., Petrov M., Huang N., Guiderdoni E., and Ghesquiere A. (1996). Aroma in rice: genetic analysis of a quantitative trait. *Theoretical and Applied Genetics*, 93: 1145–1151.

Ma H., Yin Y., Guo Z. F., Chen L. J., Zhang L., Zhong M., and Shao G. J. (2011). Establishment of DNA fingerprinting of Liaojing series of Japonica rice. *Middle-East Journal of Scientific Research,* 8(2): 384–392.

Nadaf A. B., Krishnan S., and Wakte K. V. (2006). Histochemical and biochemical analysis of major aroma compound (2-acetyl-1-pyrroline) in Basmati and other scented rice (*Oryza sativa* L). *Current Science*, 91: 1533–1536.

Nagaraju J., Kathirvel M., Kumar R. R., Siddiq E. A., and Hasnain S. E. (2002). Genetic analysis of traditional and evolved Basmati and non-Basmati rice varieties by using fluorescence-based ISSR-PCR and SSR markers. *Proceedings of the National Academy of Sciences of the United States of America*, 99: 5836–584.

Palanga K., Traore K., Bimpong K., Jamshed M., and Mkulama M. (2016). Genetic diversity studies on selected rice varieties grown in Africa based on aroma, cooking and eating quality. *African Journal of Biotechnology,* 15(23): 1136–1146.

Perez C. M., and Juliano B. O. (1988). Varietal differences in quality characteristics of rice layer cakes and fermented cakes. *Cereal Chemistry*, 65: 40–43.

Pico Y., Fernandez M., Ruiz M. J., and Font G. (2007). Current trends in solid-phasebased extraction techniques for the determination of pesticides in food and environment. *Journal of Biochemical and Biophysical Methods,* 70: 117–131.

Powel W., Morgante M., Andre C., Hanafey M., Vogel J., Tingey S., and Rafalski A. (1996). Comparison of RFLP, RAPD, AFLP and SSR markers for germplasm analysis. *Molecular Breeding*, 2(3): 225–238.

Powell W., Morgante M., Andre C., Hanafey M., Vogel J., Tingey S., and Rafalski A. (1996). Comparison of RFLP, RAPD, AFLP and SSR markers for germplasm analysis. *Molecular Breeding,* 2(3): 225–238.

Prathepha P. (2012). Genetic diversity and population structure of wild rice, *Oryza rufipogon* from North Eastern Thailand and Laos. *Australian Journal of Crop Science,* 6(4): 717–723.

Ribeiro-Carvalho C., Guedes-Pinto H., Igrejas G., Stephenson P., Schwarzacher T., and Heslop-Harrison J. S. (2004). High levels of genetic diversity throughout the range of the Portuguese wheat landrace 'Barbela. *Annals of Botany,* 94(5): 699–705.

Sajib A. A., Bhuiya M. A. I., and Huque R. (2017). A simple, efficient and rapid method for good quality DNA extraction from rice grains. *Rice Science*, 24(2): 119–122.

Sajib A. M., Musharaf Hossain Md., Mosnaz A. T. M. J., Hossain H., Monirul Islam Md., Shamsher Ali Md., and Prodhan S. H. (2012). SSR marker-based molecular characterization and genetic diversity analysis of aromatic landreces of rice (*Oryza sativa* L.). *Journal of BioScience and Biotechnology*, 1(2): 107–116.

Singh R. K., Singh U. S., and Khush G. S. (2000). Aromatic rice. Oxford and IBH publishing Co. PVT. Ltd., New Delhi.

Singh V., Roychand P., Brar M. S., and Malhi S. S. (2010). Arsenic in water, soil, and rice plants in the indo-gangetic plains of northwestern India. *Communications in Soil Science and Plant Analysis*, 41(11): 1350–1360. DOI: 10.1080/00103621003759353.

Soria A. C., Sanz J., and Martinez-Castro I. (2009). SPME followed by GC–MS: A powerful technique for qualitative analysis of honey volatiles. *European Food Research and Technology*, 228: 579–590.

Sriseadka T., Wongpornchai S., and Kitsawatpaiboon P. (2006). Rapid method for quantitative analysis of the aroma impact compound, 2-acetyl-1-pyrroline, in fragrant rice using automated headspace gas chromatography. *Journal of Agricultural and Food Chemistry*, 54: 8183–8189.

Stashenko E. E., and Martinez J. R. (2007). Sampling volatile compounds from natural products with headspace/solid-phase micro-extraction. *Journal of Biochemical and Biophysical Methods,* 70: 235–242.

Sun S. X., Gao F. Y., Lu X. J., Wu X. J., Wang X. D., Ren G. J., and Luo H. (2008). Genetic analysis and gene fine mapping of aroma in rice (*Oryza sativa* L. Cyperales, Poaceae). *Genetics and Molecular Biology*, 31: 532–538.

Tava A., and Bocchi S. (1999). Aroma of cooked rice (*Orvza sativa*): Comparison between commercial Basmati and Italian line 135–3. *Cereal Chemistry,* 76: 526–529.

Temnykh S., Park W. D., Ayres N., Cartinhour S., Hauck N., Lipovich L., Cho Y. G., Ishii T., and McCouch S. R. (2000). Mapping and genome organization of microsatellite sequences in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics,* 100(5): 697–712.

Widjaja R., Craske J. D., and Wootton M. (1996). Comparative studies on volatile components of non-fragrant and fragrant rices. *Journal of the Science of Food and Agriculture*, 70: 151–161.

Wongpornchai S., Sriseadka T., and Choonvisase S. (2003). Identification and quantitation of the rice aroma compound, 2-acetyl-1- pyrroline, in bread flowers (Vallaris glabra Ktze). *Journal of Agricultural and Food Chemistry*, 51: 457–462.

Yoshihashi T. (2002). Quantitative analysis on 2-acetyl-1-pyrroline of aromatic riceby stable isotope dilution method and model studies on its formation duringcooking. *Journal of Food Science,* 67(2): 619–622.