

Rapid Gas Chromatographic Technique for Quantifying 2-Acetyl-1-Pyrroline and Hexanal in Rice (*Oryza sativa*, L.)

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ABSTRACT

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The aroma of rice plays a role in its consumer acceptability. The popcorn-like smell of aromatic rice stemming primarily from its 2-acetyl-1-pyrroline (2-AP) content is considered desirable by many consumers. Conversely, hexanal has been correlated with off odors in rice that develop from lipid oxidation. A rapid method for 2-AP and hexanal quantification suitable for use in breeding programs, large-scale research efforts, and quality assurance programs is needed. While developing such a method, sample preparation (degree of milling, particle size), solvent extraction time and temperature, and gas chromatographic parameters were studied. Particle size had no influence on 2-AP or hexanal recovered. One extraction solubilized ≈80% of the 2-AP and 56% of the hexanal present

in milled rice. The optimum extraction method was assessed to require 0.3 g of ground brown or milled rice in methylene chloride held at 85°C for 2.5 hr. The complete gas chromatographic run requires ≈25 min, and 50 samples can be analyzed per day. The optimized method's linear response ($R^2 = 0.99$) and reproducibility was demonstrated. The stability of 2-AP and hexanal in frozen milled rice and in refrigerated methylene chloride extracts was excellent for at least six months. Milled and unmilled commercial and breeders' aromatic rice samples contained 10–1,104 ng/g of 2-AP and 148–2,541 ng/g of hexanal. Genotype had the greatest effect on the 2-AP and hexanal content of two lines grown over four years and in four states.

More than 100 compounds that contribute to the aroma of rice (*Oryza sativa* L.) have been identified (Tsugita 1986, Widjaja et al 1996). Some of these volatile compounds contribute to consumer acceptance of certain types of rice, whereas other compounds contribute to consumer rejection. The two issues currently of greatest importance to the industry are the aroma of aromatic rice and off flavors due to rancidity.

The aromatic compound 2-acetyl-1-pyrroline (2-AP) is the primary component responsible for aromatic (scented) rice aroma (Buttery et al 1983, 1988). Consumption of aromatic rice has increased in the United States during the last decade, and U.S. rice imports have consisted primarily of aromatic rice types, specifically those known as Jasmine and Basmati. In 1997–98, ≈214,000 metric tons of milled Jasmine-style rice was imported from Thailand and ≈45,000 metric tons of milled Basmati-style rice was imported from India and Pakistan. That is equivalent to ≈4.3% of U.S. production; the value of those imports was ≈\$178 M, up from \$46 M in 1987–88 (U.S. Department of Commerce 1998).

Efforts are ongoing by several U.S. rice breeding programs to develop aromatic rice cultivars that are agronomically acceptable to producers and have end-use properties comparable to imported aromatic rice, thus providing an opportunity for U.S.-produced aromatic rice to compete with imported rice. Consequently, rapid methods focused on aromatic rice quality are needed to assist rice breeders in making their selections from the multitude of lines they evaluate each year. Such methods would also make possible projects requiring large numbers of samples, such as genetic and storage studies.

The scent of aromatic rice is a highly heritable trait and reportedly is under the control of one to four genes, depending on the population studied (Dhulappanavar 1976, Tripathi and Rao 1979, Reddy and Sathyanarayanaiah 1980, Pinson 1994). Tsuzuki and Shimokawa (1990) suggested that the lack of agreement regarding the number of genes controlling aroma may be, in some instances, a consequence of the empirical methods used to assess rice aroma. Examples of those methods include biting kernels, smelling vegetative tissue after warming or soaking in KOH, and eating cooked rice (Dhulappanavar 1976, Sood and Siddiq 1978, Tripathi and Rao 1978). Sensory fatigue is likely a source of error for such methods.

Popcorn, leavened bread, pandan leaves, and wet millet reportedly contain 2-AP (Buttery et al 1982; Schieberle 1990, 1991, Seitz et al 1993). Agronomic and postharvest practices influence the levels of 2-AP in aromatic rice (Goodwin et al 1994a,b). However, there is only limited evidence that there are genetic differences associated with the intensity of 2-AP (Pinson 1994). There have been no reports on the metabolic pathway that leads to the synthesis of this compound.

Quantification of 2-AP in rice has been performed by Buttery et al (1986) and Lin et al (1990) using a simultaneous steam distillation and solvent extraction apparatus and 100–200 g of sample. Tanchotikul and Hsieh (1991) using a microsteam distillation and solvent extraction procedure extracted 2-AP from 1-g samples and noted that eight samples could be done per day. The extraction time for these methods renders them impractical for use in breeding programs.

Other volatiles associated with a specific aspect of rice aroma are the carbonyl compounds. They cause stale or rancid odors in many foods (Bennion 1980). Rice lipids are primarily in the bran and germ. Therefore, development of rancidity is of greatest concern for industries focused on brown rice, rice bran, or its derivatives. However, even with <1% lipid, milled rice also is reportedly susceptible to developing oxidative rancidity during storage (Sowbhagya and Bhattacharya 1976). Consequently, efforts have been made to define storage conditions and processing parameters intended to minimize lipid oxidation (Mitsuda et al 1972, Sowbhagya and Bhattacharya 1976, Ory et al 1980, Sharp and Timme 1986, Champagne and Hron 1993). Lipase and lipoxygenase activity are thought to be associated with rice susceptibility to lipid oxidation, but the genetic and environmental effects controlling rancidity are not understood.

Increased levels of carbonyl compounds are associated with lipid deterioration. Of those compounds, hexanal increases the most during rice storage and consequently has been used as an indicator of

¹ USDA, ARS, Rice Research Unit, 1509 Aggie Drive, Beaumont, TX 77713. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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rancidity (Yasumatsu et al 1966, Shibuya et al 1974, Tsugita et al 1983, Gon Shin et al 1986, Champagne et al 1993). The methods used during those storage studies to quantify hexanal are precise but relatively time-consuming, and thus not suitable for large research projects, breeding programs, or quality assurance departments.

The objective of this project was to develop and evaluate a method for quantifying rice 2-AP and hexanal that is relatively inexpensive, requires minimal sample size, and has a fast throughput.

MATERIALS AND METHODS

Rough rice samples of Della grown in 1993 (DLA93) and 1994 (DLA94), and Dellmont grown in 1994 (DLMT94), obtained from one replicate of the Uniform Rice Regional Nursery (URRN) grown in Beaumont, TX, were used to develop the method for 2-AP and hexanal. The gas chromatography (GC) method reported here was used to examine Della and a breeding line (RU9802177) grown in Arkansas, Louisiana, Mississippi, and Texas, in the URRN, from 1994 to 1997. The samples were hulled on a rice huller (type THO, Satake, Houston, TX), and milled using a grain testing mill (model TM05, Satake) at 1,250 rpm, using a 36-mesh abrasive wheel. Broken kernels were removed using a No. 12 sizing screen. The GC method was also used to examine commercial aromatic rice samples that were purchased in retail stores.

Sample Extraction

The extraction vials used were 12 × 32 mm with a TFE septa and crimp top. Extractions were performed using (unless otherwise noted) 0.3 g of ground rice in 0.5 mL of stock solution (Sarreal et al 1997). Samples were ground to 20 mesh (Wiley mill, model 475A). The stock solution consisted of methylene chloride (Optima

grade, Fisher Scientific) with 0.459 ng/mL of 2,4,6-trimethylpyridine (TMP: Collidine, Aldrich) used as an internal standard.

The effect of extraction temperature (45, 80, 85, and 90°C) in a circulating water bath, sonication (at room temperature), and extraction time (0.5, 1.5, 2.5, 3.5, and 4.5 hr) on 2-AP and hexanal recovery was investigated using DLA93. Extraction procedure efficiency was examined using DLA94 in the brown rice form and after milling for 1.5, 4, and 10 min. Samples were extracted four times, the extraction liquid was pipetted off after each extraction and the rice rinsed two times with methylene chloride before reextraction. Optimum sample particle size was studied by analyzing the 2-AP and hexanal content of DLA94 ground using the Wiley mill fitted with a 20, 40, 60, or 80-mesh screen. The reproducibility of the extraction procedure deemed to be optimum was assessed by subsampling and extracting DLMT94 (stored at 4°C) 30 times over a six-month period. Each subsample of frozen milled rice was allowed to equilibrate to room temperature before grinding.

Gas Chromatography

Method development and sample analysis were performed on a HP-5890 Series II gas chromatograph (GC) with HP-3365 ChemStation using a DB-5 column (0.53 i.d., 30 m, J&W Scientific) and flame-ionization detection (FID). After optimization, the GC operating parameters were chosen: injector 155°C (splitless with a 30-sec valve delay); detector 300°C; initial temperature 35°C; initial time 1 min, first rate 9°C/min to 120°C, second rate 25°C/min to 275°C; final temperature 275°C; final time 2 min. Helium was the carrier gas and was set at a constant flow rate of 7.2 mL/min. A volume of 2 µL of extract was injected. The time required to analyze the peaks of interest and purge and cool the system was 25 min.

The 2-AP peak was originally identified by chromatography-coupled aroma perception with simultaneous FID similar to the procedure described by Tanchotikul and Hsieh (1989). R. Buttery of USDA, ARS, WRRC in Albany, CA, kindly provided synthesized 2-AP for verification of the method's linearity and ana-

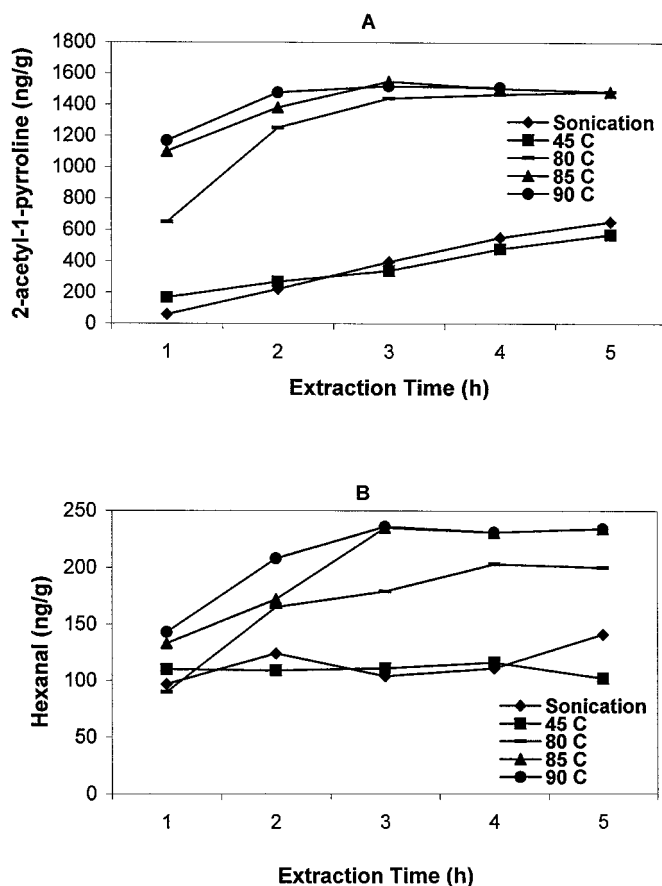


Fig. 1. Effect of extraction time, temperature, and sonication on 2-acetyl-1-pyrroline (A) and hexanal (B) recovery from an aromatic rice. As-is basis.

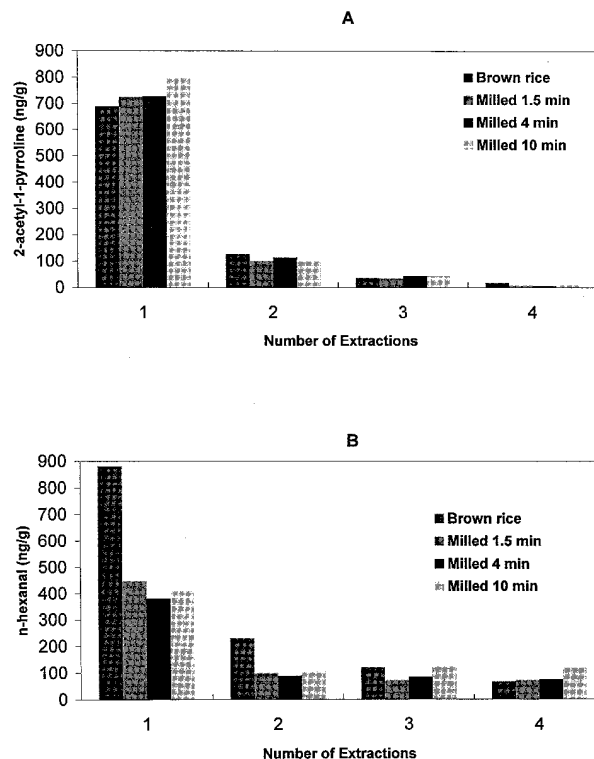


Fig. 2. Effect of degree of milling and number of extractions on 2-acetyl-1-pyrroline (A) and hexanal (B) recovery from aromatic rice. As-is basis.

lytical reagent-grade hexanal was used for its verification (Capronaldehyde, Fluka). Method linearity for 2-AP and hexanal were also examined by analyzing DLLA93 at several different sample weights. Mass spectra for 2-AP and a second peak associated with aromatic rice were determined using an HP-5973 GC/mass spectrometer. The GC conditions were the same as above. The mass spectrometer (MS) was operated in scan mode at 50–400 m/z using electron ionization.

Routine quantification of 2-AP and hexanal was performed by calculating the detector response relative to that for TMP (Buttery et al 1986). To determine recovery of TMP, unextracted stock solution TMP area counts were compared with TMP area counts of extracted stock solution and with TMP area counts of the extracted rice samples. The results of that comparison indicate that minimal or no loss of TMP occurs during the extraction procedure. The repeatability of the GC analysis was examined using DLMT94. Triplicate samples (2.5 g) of milled rice were extracted with stock solution (5 mL), supernatants were combined, and aliquots dispensed into sealed vials for refrigerated storage. A total of 30 GC analyses of this pooled extract were performed over a six-month period.

Statistical Analysis

Analysis of variance (ANOVA) and Fisher's least significant difference (LSD) were performed using SAS version 7 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

The extraction method initially developed during this project consisted of extracting 2.5 g of milled rice with methylene chloride in 13 × 100 mm screw cap test tubes for 3 hr. After centrifugation, 3.0 mL of the clear supernatant was filtered through a sodium sulfate drying tube. The filtrate was evaporated to 0.2 mL using a stream of nitrogen, and then 3 μL was injected on to a DB-5 column. Results using that extraction method were comparable to those obtained using the faster method described below.

The minimum extraction time that achieved maximum 2-AP and hexanal recovery was 2.5 hr at 80–90°C (Fig. 1). The temperature of 85°C was chosen as the standard extraction temperature because some loss of vial content occurred at the higher temperature due to septum failure. Extracting in a sonicator resulted in values similar to those obtained at 45°C without sonication. The actual temperature in the sonicator was ≈50°C due to heat gained from the sonicator. Sonication appeared to have no effect on 2-AP or hexanal extraction.

The efficiency of consecutive extractions is displayed in Fig. 2. Regardless of milling degree, 80–85% of the total 2-AP was extracted on the first extraction. The second extraction removed another 10–12%. By the fourth extraction, recovery of 2-AP was at the limit

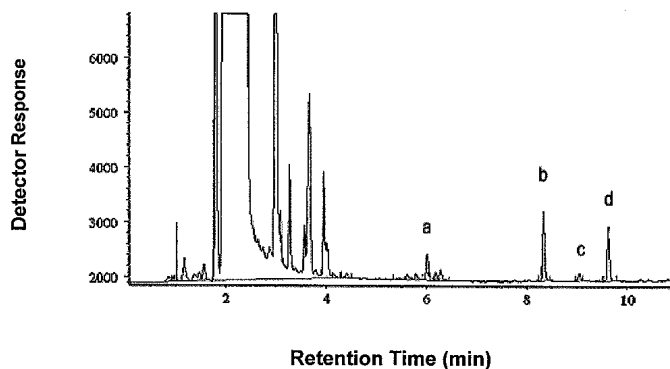


Fig. 3. Chromatogram of typical aromatic rice extract. Peak a, hexanal; b, 2-acetyl-1-pyrroline; c, aromatic rice associated peak; and d, 2,4,6-trimethylpyridine (TMP).

of peak detection. No difference in 2-AP levels between the brown and milled rice samples suggests that this compound is equally distributed in the bran and endosperm.

After one extraction, longer milling time was associated with lower hexanal recovery. The most probable reason for this outcome is that the milling process removed the lipid-rich bran layer, leaving the endosperm with lower levels of hexanal and other lipid breakdown products. The brown rice and the sample milled for 10 min had 68 and 54% recovery of hexanal, respectively, after one extraction. Those values are greater than that reported to be a reasonable quantitative recovery of food volatiles (Buttery et al 1988). With that in mind, and in the interest of having a quick method, it was decided that one extraction was sufficient to determine relative quantities of 2-AP and hexanal. However, if a total accounting of the quantity of 2-AP or hexanal in a sample is needed, a recovery factor should be used to adjust the values.

Although particle size did not have a significant effect on the quantity of 2-AP or hexanal extracted (data not shown), the recovery of 2-AP and hexanal from whole kernel rice was ≈50% less than that from the ground samples. Our studies using whole kernel rice indicate that adding water to the extraction solution results in 2-AP recoveries comparable to the ground rice results.

Based on the results of the extraction studies, the procedure chosen includes sample grinding using a Wiley mill with 20-mesh screen and one methylene chloride extraction step at 85°C for 2.5 hr. A typical chromatographic separation achieved using a methylene chloride extract of aromatic rice is displayed in Fig. 3. The reten-

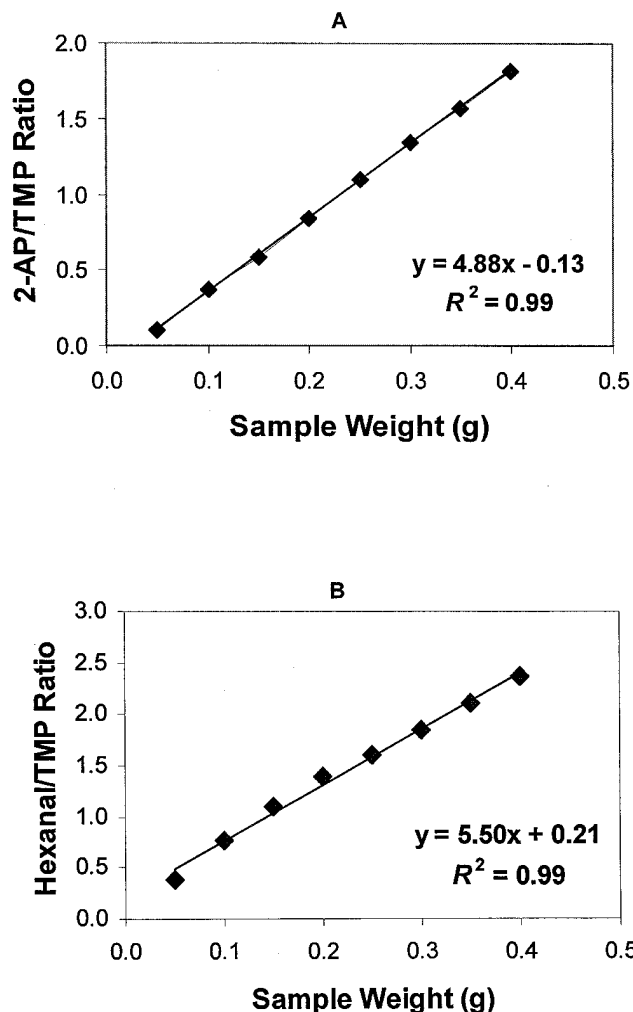


Fig. 4. Linear response of 2-acetyl-1-pyrroline (2-AP) (A) and hexanal (B) using various amounts of aromatic rice. As-is basis. TMP = 2,4,6-trimethylpyridine.

tion time for the monitored peaks are hexanal at 6.13 min, 2-AP at 8.47 min, and TMP at 9.77 min. The method was linear using 2-AP ($\leq 2,225$ ng/ μ L) and hexanal (up to 4,000 ng/ μ L) external standards (data not shown) and various amounts of an aromatic sample (Fig. 4).

The repeatability of the GC method using extract stored under refrigeration for six months had a coefficient of variation of 3.1% and a standard error of 5.0. The results for the frozen rice subsamples analyzed during six months had a coefficient of variation of 7.9%, and a standard error of 14. Those values are similar to the level of repeatability reported by Tanchotikul and Hsieh (1991) for a 2-AP method using a steam distillation extraction. The extraction procedure and GC quantification method were thus deemed to have satisfactory repeatability. The results also demonstrate the stability of 2-AP and hexanal in refrigerated extract and frozen milled rice.

We have analyzed >2,000 aromatic and 200 nonaromatic rice samples using this method. A peak with a retention time of 9.18 min was found in all of those aromatic rice samples but not in any nonaromatics (Fig. 3). We found no reports indicating that any com-

pound, other than 2-AP, is unique to aromatic rice. Upon GC/MS analysis, this peak was tentatively identified as an isomer of 2-AP (Fig. 5). The 2-AP isomer has the same molecular ion at m/z 111 and undergoes the loss of HCN to produce fragments at m/z 83, analogous to the 2-AP fragmentation pattern. One notable difference is the base peak of the 2-AP isomer at m/z 55, while for 2-AP it is at m/z 83. Based upon this information and the likelihood of formation, this isomer may contain the double bond between the number two and three carbons rather than between the nitrogen and the number two carbon (R. Buttery, *personal communication*). Work is in progress to verify these conclusions and to determine whether the 2-AP isomer adds to the scent of aromatic rice.

The utility of this method is demonstrated by examining the results from two aromatic rice genotypes grown in the URRN over four years. The values for 2-AP and hexanal in those samples were 251–1,105 and 202–1,104 ng/g, respectively. Main effects and interactions significantly affected 2-AP and hexanal levels (Table I). Genotype contributed the greatest amount of variation for 2-AP and hexanal. That is surprising, considering that the source of aroma in the breeding line's pedigree is Della (Farmen Jodari, *personal communication*). Thus, Della and the breeding line have the same recessive gene reported by Berner and Hoff (1986) controlling their aroma content, yet their actual levels of 2-AP differ. The genetic background of this gene may influence its expression or affect the level or activity of other compounds that are involved in the synthesis or retention of 2-AP. No previous reports of genetic versus environmental effects on hexanal levels in rice were found.

TABLE I
Mean Squares for Combined Analysis of Variance
Across Genotypes, Years, and States^a

Variation	df	2-Acetyl-1-pyrroline	Hexanal
Genotype (G)	1	2,234,277	785,660
Year (Y)	3	75,161	441,348
State (S)	3	138,042	105,928
G \times Y	3	278,167	140,358
G \times S	3	30,331	33,776
Y \times S	9	19,431	27,787
G \times Y \times S	9	433,703	229,332

^a All sources of variation were significant at $P < 0.01$.

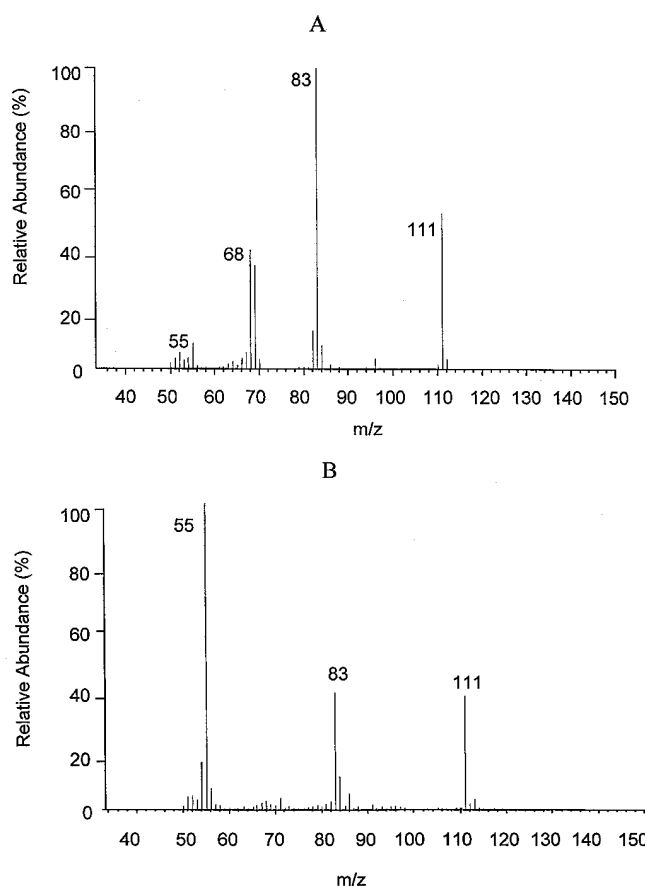


Fig. 5. Mass spectrum for 2-acetyl-1-pyrroline (A) and putative 2-acetyl-1-pyrroline isomer (B) in aromatic rice.

TABLE II
Genotype, Year, and State Means^a

	2-Acetyl-1-pyrroline	Hexanal (ng/g)
Genotype		
Della	860a	592a
RU9802177	486b	371b
Year		
1994	676b	705a
1995	644c	491b
1996	767a	414c
1997	606d	314d
State		
Arkansas	605c	585a
Louisiana	731b	494b
Mississippi	583d	457c
Texas	772a	390d

^a Means within the categories of genotype, year, or state followed by the same letter are not significantly different ($P < 0.05$) based on Fisher's least significant difference test.

TABLE III
Concentration of 2-Acetyl-1-pyrroline (2-AP) and Hexanal
in Various Types of Rice Obtained from Retail Stores^a

Rice Type (Company)	Rice Form	2-AP (ng/g)	Hexanal (ng/g)
Basmati (Tilda)	Milled	342	1,412
Basmati Easy Cook (Tilda)	Milled	19	543
Basmati (Fantastic Foods)	Milled	68	1,175
Basmati (Fantastic Foods)	Brown	119	1,416
Basmati (Vigo)	Milled	149	2,209
Basmati (Sharda)	Milled	131	987
Jasmine (Fantastic Foods)	Brown	550	1,282
Jasmine (Millagrosa MRR)	Milled	598	776
Jasmine (ITC)	Milled	810	922
Black Thai (bulk)	Brown	259	2,541
Aromatic (Fowler Gourmet)	Milled	999	717
Amber aromatic (Lundberg)	Brown	345	1,366
Kasmati (Rice Tec)	Milled	496	1,203
Texmati (Rice Tec)	Milled	266	1,240
Jasmati (Rice Tec)	Milled	526	954
LSD ^b		80	148

^a Values are means of two replicates and reported on an as-is basis.

^b Least significant difference ($P < 0.05$).

The second largest amount of variation in 2-AP was from genotype-year-state interaction effects, and for hexanal, it was from year effects. Mean separation across years and states showed that Della had more 2-AP and hexanal compared with the breeding line (Table II). Hexanal values were greatest in Arkansas and lowest in Texas. More southern growing regions (Texas and Louisiana) had greater 2-AP levels compared with more northern areas (Arkansas and Mississippi). Goodwin et al (1994b) reported that the cultivar Jasmine 85 had greater 2-AP levels from grain grown in Mississippi compared with Texas, Louisiana, or Arkansas during 1992 and 1993. These conflicting results may be an indication of the influence of agronomic practices or genotype and environment interactions on 2-AP levels. Lack of agreement between studies and finding two- and three-way interaction effects on 2-AP levels to be significant in the present study explains, in part, why breeding for this trait is such a challenge and why a rapid quantification method is important.

The merit of this GC method is also shown by the results found from a survey of commercial aromatic rice samples obtained from U.S. and Thai retail markets. Those samples had 2-AP and hexanal levels of 19–999 and 543–2,541 ng/g, respectively (Table III). Using this GC method, 2-AP was not detectable in nonaromatic commercial rice samples. No reports of hexanal values in commercial aromatic or nonaromatic rice samples could be found. Buttery et al (1983) reported being able to detect 2-AP (<0.008 ppm) in two U.S. nonaromatic rice commercial samples using a steam distillation extraction system. The 2-AP content of three commercial basmati-type rice samples examined by Sarreal et al (1997) was <208 ng/g.

CONCLUSIONS

Using this method, an analyst could determine 2-AP and hexanal in ≈50 rice samples per day. Also, the method is relatively inexpensive as the GC run is <25 min and no special extraction device is required.

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