# An Improved Method for Quantification of 2-Acetyl-1-pyrroline, a "Popcorn"-like Aroma, in Aromatic Rice by High-Resolution Gas Chromatography/Mass Spectrometry/Selected Ion Monitoring

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A rapid microscale steam distillation/solvent extraction procedure was used to extract parts per billion (nanograms per gram) levels of a "popcorn"-like aroma compound, 2-acetyl-1-pyrroline, from milled aromatic rice (*Oryza sativa*, L.) samples. Improvements on the gas chromatographic separation and mass spectrometric sensitivity and specificity in the selected ion monitoring mode required only 1 g of rice for each analysis. Selected aromatic rice samples, including Della, Basmati 370, and Jasmine, were found to contain 2-acetyl-1-pyrroline in the range 76–156 ppb on the basis of equivalent weight of an internal standard, 2,4,6-trimethylpyridine, and dry weight of rice.

Rice is a major food commodity and is consumed worldwide. Aromatic or scented rice has been popular in the Orient and is becoming more popular in Europe (Berner and Hoff, 1986) and the United States (Brooks, 1989). Aromatic rice is often preferred over the nonscented variety due to its pleasant aromas (Reddy and Reddy, 1987) and commands a high market value.

A "popcorn"-like aroma compound, 2-acetyl-1-pyrroline (AP), has been reported as an important flavor component of several aromatic rice varieties because of its much higher odor potency than the other volatile components in rice (Buttery et al., 1983; 1988). Buttery et al. (1983) extracted AP and other volatile components from 500-g rice samples by using a Likens–Nickerson (Likens and Nickerson) (1964) simultaneous steam distillation/solvent extraction (SDE) apparatus. The extracts were analyzed by gas chromatography/mass spectrometry (GC/MS) in a full MS scan mode for identification (Buttery et al., 1983). Buttery et al. (1986) used SDE to extract 200 g of rice for quantification of AP by GC–flame ionization detection (FID). Lin et al. (1990) also used a similar preparative-scale SDE procedure to extract aroma compounds from 200-g rice samples for identification and quantification of AP in Louisiana Della rice by GC/MS in a full-scan mode.

Efforts in breeding of aromatic rice can be greatly facilitated by establishing the concentrations of the aroma compounds in a new cultivar. However, only a small number of seeds can be made available during the early breeding period. A quantity of 200 g, equivalent to approximately 12 000 milled grains, often exceeds what a rice breeder is willing to commit to a destructive test. An improved quantitative method using a small amount of rice sample must be established to fulfill rice breeders' needs.

Chromatographic interference problems have been reported by Paule and Powers (1989) using a packed column. Coeluting compounds, if not excluded, can often contribute to errors in overestimating the quantity of an analyte.

The objective of this study was to develop a more sensitive procedure for quantification of 2-acetyl-1-pyrroline in aromatic rice samples using small (low gram) quantities with emphasis on improved chromatographic separation and detection specificity.

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## MATERIALS AND METHODS

**Materials.** Milled Della (1989 crop), Jasmine (1989 crop), and Basmatic 370 (1988 crop) rice samples were obtained from the Rice Research Station of the Louisiana Agricultural Experiment Station in Crowley, LA. The samples were stored at −20 °C in amber bottles before analysis. The rice samples were ground by using a grinder (Model Galaxie, Oster Corp., Milwaukee, WI) and passed through a 35-mesh sieve.

An antifoam solution with minimized levels of volatile compounds was prepared by adding 20 mL of antifoam (catalog no. A-5757, Sigma Chemical Co., St. Louis, MO) to 600 mL of water and concentrating the mixture to 200 mL by boiling according to the method of Buttery et al. (1986).

Standard 2-acetyl-1-pyrroline (AP) was obtained from Dr. R. Buttery of USDA ARS WRRC in New Albany, CA. The compound 2,4,6-trimethylpyridine (TMP, density 0.917, 99% purity), was purchased from Aldrich Chemical Co. (Milwaukee, WI) and used as an internal standard.

**Extraction of AP from Rice Samples.** The AP was extracted from rice samples by using a microscale steam distillation/low-density solvent extraction device (micro-SDE, catalog no. 16050, Chrompack, Raritan, NJ). Ground rice samples (1, 2, or 3 g), 100 mL of distilled water, 2 mL of antifoam solution, 4 mL of 25 ng/mL TMP solution in water, and 18 glass beads (3 mm i.d.) were placed in a 100-mL round-bottom sample flask. Four milliliters of redistilled diethyl ether was added to a 9-mL pear-shaped solvent flask. Both the sample and solvent flasks were attached to the appropriate arm of the apparatus. Distilled water was added to the separation chamber before it flowed over the water return tube. Then, diethyl ether was added to the same chamber until it was about to flow out of the solvent return tube. The cold finger was cooled to −5 °C with a refrigerated circulating bath (Model RTE110B, Neslab Instruments, Inc., Portsmouth, NH). The two vapor transport arms and the solvent flask were heated with heating tapes (catalog no. B 00051020, Thermolyne Corp., Dubuque, IA) controlled by a temperature controller (catalog no. PL 312, Glas-Col, Terre Haute, IN) with the dial set at position 2. The sample flask was heated in a heating mantle (catalog no. MG 7803, Electrothermal, England) controlled with a variable autotransformer (Type 3PN1010, Staco Energy Products Co., Dayton, OH). The autotransformer was initially set at 80% of maximum output voltage and changed to 60% after the sample began to boil at approximately 102 °C to avoid overheating and bumping in the flask. The distillation/extraction continued for 2 h after the sample started to boil. The solvent

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flask was then detached from the device, and the ether layer was dried over 5 g of anhydrous sodium sulfate. Triplicate extractions were carried out for each sample.

**Gas Chromatography/Mass Spectrometry (GC/MS).** The volume of each extract was reduced to 0.2 mL under nitrogen. Five microliters of the 0.2-mL extract was injected onto a fused silica capillary column (Supelcowax 10; 0.25 mm i.d. × 60 m length × 0.25 μm film thickness; Supelco, Inc., Bellefonte, PA) installed in a Hewlett-Packard (HP) 5792A gas chromatograph (Palo Alto, CA). Helium gas (purity 99.999%, passed through a molecular sieve and an oxygen trap) at a linear velocity of 25 cm/s was used as the GC carrier gas. The injector and the GC/MS interface temperatures were set at 155 and 195 °C, respectively. A hexane solution containing each of the standard compounds [*n*-tridecane, (*E*)-2-heptenal, AP, 6-methyl-5-hepten-2-one, 1-hexanol, TMP, and *n*-tetradecane] was chromatographed at a level of approximately 30 ng/component under various GC oven temperature conditions to optimize chromatographic separation. The two *n*-alkanes were included for the calculation of retention index (Kovats, 1958). 1-Hexanol has been reported by Paule and Powers (1989) as an interference compound for AP. (*E*)-2-Heptenal and 6-methyl-5-hepten-2-one have been observed in rice extracts with elution times close to that of AP (Lin, unpublished data). After optimization, the following column oven temperature program was chosen for subsequent analyses: the column temperature was maintained isothermally at 65 °C for 70 min and then programmed at a rate of 8 °C/min to 195 °C and kept at 195 °C for 15 min. An HP 5970B mass selective detector (MSD) was used in the electron ionization (EI) mode with the ion source temperature set at 200 °C, ionization energy at 70 eV, and electron multiplier voltage at 2400 V. Selected ion monitoring (SIM) was set up to monitor two groups of characteristic ions: group 1 ions, $m/z$ (mass/charge) 68, 69, 82, 83, 84, 108, 111, and 112 from retention time 30–47 min for AP; and group 2 ions, $m/z$ 51, 53, 77, 79, 106, 120, and 121 from retention time 47–55 min for TMP. MS detection dwell time was 100 ms for each ion. Ion abundance ratios of the analyte AP and internal standard TMP were compared with corresponding ratios in full-scan spectra of standard AP and TMP to ensure correct selection of GC peaks. To enhance detection specificity with minimum interference, the sum of peak areas of $m/z$ 68, 83, and 111 at retention index of 1331 was integrated for AP. For the peak area of TMP, the peak of $m/z$ 121 at the retention index of 1356 was used. Each extract was analyzed twice by GC/MS to obtain an average peak area.

**Determination of the Conversion Factor from SIM to Full-Scan MS Detection.** Each of the AP and TMP standard solutions was injected and detected in the SIM and the full-scan ($m/z$ 40–122) mode. The conversion factor for AP from SIM to full-scan MS detection was calculated by dividing the peak area in the full-scan mode over the peak area in the SIM mode. The conversion factor for TMP from SIM to full scan was calculated in a similar manner. Two injections were made for each compound in each mode to obtain average values.

**Confirmation of the Aroma Characteristics of the AP Peak.** Chromatography-coupled aroma perception analysis with simultaneous photoionization detection as described by Tanchotikul and Hsieh (1989) was used to confirm the popcorn-like aroma in the GC effluent from the sample extracts at the expected retention index. GC conditions were the same as those used in GC/MS analyses except that an HP5793 GC was used.

**Determination of Linearity and Sensitivity.** Although the standard AP solution contained an amount of AP sufficient for positive identification, the solution had undergone considerable degradation, and the absolute purity of the AP was uncertain. To our knowledge, pure AP was not available elsewhere. The linearity and sensitivity of this method were thus demonstrated by analyzing Della rice at 1-, 2-, and 3-g levels instead of spiking exact amounts of AP to a control (nonaromatic) rice sample.

**Determination of Relative Recovery Factor (RRF).** A precise amount of standard mixture of AP and TMP was spiked into a 100-mL round-bottom flask containing 2 mL of antifoam solution, 100 mL of distilled water, and 18 glass beads. The micro-SDE operation was performed in the same manner as mentioned above. To maintain consistent boiling and avoid bumping in this mixture, the Staco controller temperature for the sample flask was set at 60% initially and decreased to 45% after boiling. Triplicate extractions were performed, and each extract was analyzed twice by GC/MS in the SIM mode. Separate direct injections of AP and TMP standards (without distillation/extraction) after proper dilutions also were made to obtain peak areas under the same GC and MS conditions. The relative recovery factor was calculated by dividing [(peak area of AP)/(peak area of TMP) from the extract] by [(peak area of AP)/(peak area of TMP) from direct injection of standards].

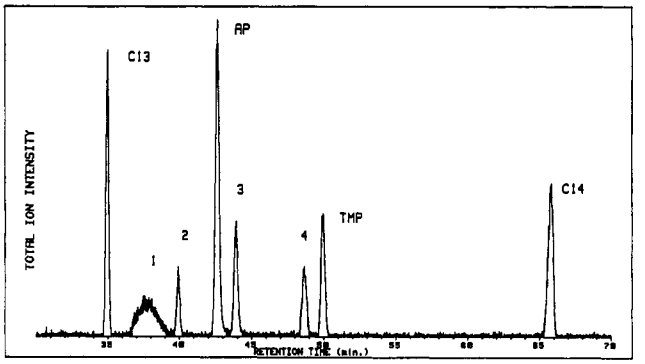


**Figure 1.** Total ion chromatogram ($m/z$ 40–290) of a test solution containing AP, TMP, *n*-tridecane, *n*-tetradecane and (peak 1) unknown, (peak 2) *trans*-2-heptenal, (peak 3) 6-methyl-5-hepten-2-one, and (peak 4) 1-hexanol.

**Calculation of Concentration of AP in Rice in Equivalent Weight of TMP.** The equation used for calculating the concentration of 2-acetyl-1-pyrroline was

$$C = \frac{RT}{W(1 - \% M)} \frac{1}{\mathrm{RRF}}$$

where $C$ is the concentration of AP (ng/g or ppb) in equivalent weight of TMP, $R$ is (peak area of AP in SIM from the extract × conversion factor of AP from SIM to full scan)/(peak area of TMP in SIM from the extract × conversion factor of TMP from SIM to full scan), which is equal to (peak area of AP in SIM from the extract)/(peak area of TMP in SIM from the extract) × 1.306, $T$ is the amount (ng) of TMP used as the internal standard (In this study, 99.0 ng of TMP was added to the sample before extraction.), $W$ is the wet weight (g) of milled rice sample, $M$ is the percent moisture content of rice sample, and RRF is the relative recovery factor (0.543 ± 0.071).

## RESULTS AND DISCUSSION

An improved analytical method was developed for quantification of 2-acetyl-1-pyrroline (AP) in milled aromatic rice. Improvements were made in the following areas: less sample weight required for analysis, better chromatographic separation, higher detection sensitivity and specificity in comparison with published procedures (Buttery et al., 1983, 1986; Lin et al., 1990).

**Extraction of AP from Rice.** The micro-SDE procedure and the GC/MS in the SIM mode allowed as little as 1 g of rice for each analysis, as compared with 200 g or more used previously (Buttery et al., 1983, 1986; Lin et al., 1990). In addition, the analysis time was reduced by more than 50%. Three extractions can be completed in about 8 h with this method. Since a smaller volume of solvent was used, the time required and the error incurred in solvent evaporation were also reduced.

**Analytical Specificity.** Paule and Powers (1989) have indicated interference of 1-hexanol with the AP peak in their work. This type of chromatographic interference might have caused certain errors in GC-coupled aroma evaluation and/or overestimation in quantification of AP. By using SIM and isothermal temperature at 65 °C for GC separation, interference from several other compounds with the AP peak (Lin, unpublished data) also was avoided as shown in Figure 1. In addition, the SIM mode produced

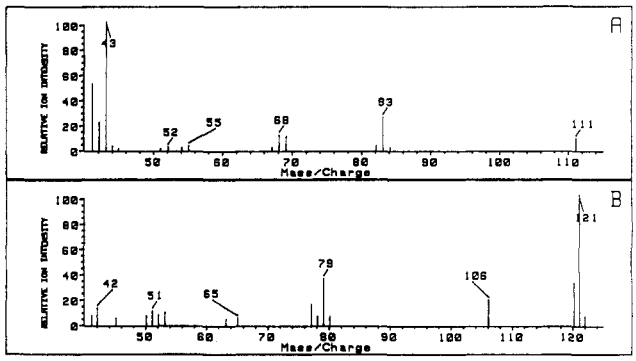


**Figure 2.** Full-scan ($m/z$ 40–122) mass spectrum of (A) AP and (B) TMP.

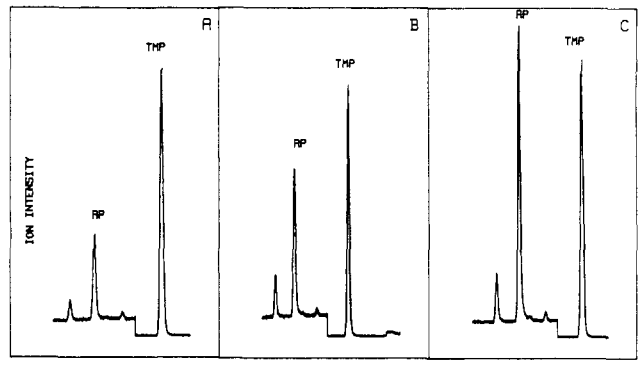


**Figure 3.** SIM chromatograms of AP and TMP of micro-SDE extract from (A) 1 g, (B) 2 g, and (C) 3 g of the Della rice sample. See Materials and Methods for the ions incorporated in tracing the chromatograms.

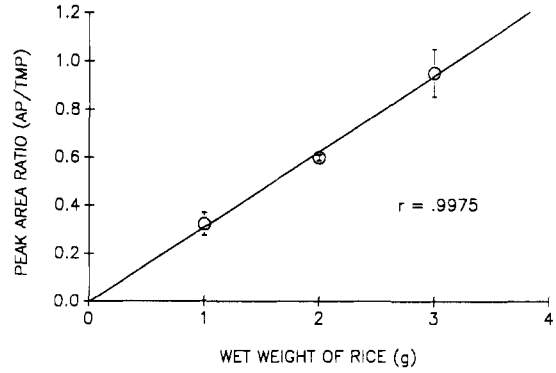


**Figure 4.** Linearity of detector response. See Materials and Methods for calculation of peak area ratios. Standard deviations of the means ($n$ = 3) are indicated by the error bars.

a much better sensitivity in comparison with the full-scan MS, since the MS dwell time for each characteristic ion is much longer in SIM than the few milliseconds for each ion in full-scan MS. Full-scan ($m/z$ 40–122) mass spectra of AP and TMP are shown in Figure 2. As described under Materials and Methods, many of the characteristic ions of these compounds were used in the SIM mode for enhanced detection sensitivity and specificity. In addition to the chromatographic retention indices, the ion abundance ratios from a total of eight ions for AP and seven ions for TMP were used to confirm the identification of AP in the SIM mode. Thus, qualitative information is still available from this highly sensitive analytical method. Finally, the peak of interest from the aromatic rice samples was detected at the same retention index of standard AP and produced the same popcorn-like aroma as that of standard AP in chromatography-coupled GC aroma perception analysis.

**Expression of AP Concentration.** Expressing the concentration of AP in equivalent weight of the internal standard TMP is proposed in this method, since TMP is relatively stable and readily available. This approach may alleviate the purity inconsistency problem due to degradation of a standard AP. This approach also will allow an easier comparison of AP concentration data from different analytical laboratories. By use of full-scan peak area ratios which were converted from SIM peak area ratios, instead of raw full-scan data, the amount of AP in equivalent weight of TMP could be more quantitatively determined. Raw data from full scan on the sample often overestimates the contribution of an analyte due to the presence of other coeluting compounds.

**Relative Recovery Factor (RRF).** Since a standard calibration curve requiring known concentrations of standard AP could not be obtained, it was necessary to correct for the difference in the recoveries for the internal standard TMP and AP extracted from the rice. The RRF as described under Materials and Methods was determined to be 0.543 ± 0.071. By incorporation of this correction factor in the calculation of AP, the value obtained for the amount of AP in the extract can be converted to the actual amount of AP present in the rice. A similar approach was reported by Buttery et al. (1986). Since an ideal nonaromatic rice matrix with the added AP distributed in a way identical with that in the aromatic rice samples was not available, and may not be available to most of the laboratories interested in performing such analysis, the relative recovery factor determined in this study would allow a simple and practical way of comparing the levels of AP in rice.

**Linearity of Detector Response.** Linear detector response was determined by analyzing 1, 2, and 3 g (wet weight) of milled Della rice. SIM chromatograms of AP and TMP at these levels of the Della rice samples are shown in Figure 3. A signal-to-noise ratio of about 3:1 was obtained at the 1-g level of Della rice. The detector response (peak area ratios of AP over TMP) was plotted against the weight of rice as shown in Figure 4. A linear regression coefficient of 0.9975 was obtained.

**Studies with Rice Samples.** Other rice samples, including Jasmine and Basmatic 370, also were analyzed to demonstrate the applicability of this method. It was found that the concentrations of AP in Della, Basmati 370, and Jasmine rice were 76.2 ± 1.8, 87.4 ± 3.4, and 156.1 ± 8.4 ppb, respectively. The results indicated the precision of the method with a percent coefficient of variation between 2.3 and 5.4 at the 2-g level of rice. The rice samples analyzed in this study were individual, isolated examples available at the time of study and were used for method demonstration only. However, meaningful comparison of concentrations among the varieties can be achieved by using this improved analytical method in conjunction with an appropriate sampling scheme designed for comparison.

This present method, requiring a sample amount of only 1–3 g of rice for quantitative determination of AP, may be helpful to rice breeders. The improved sensitivity of this method is significant, since accumulation of the number of grains at an early stage of breeding is most difficult and several seasons of cultivation time may be saved before an analysis can be performed to verify the aromatic trait. This efficient method also may be used to objectively determine the concentrations of AP in market samples of aromatic rice.

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