Annual Report
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To: California Rice Research Board

Project Title: **Development of Analytical Methods for Profiling Rice Aroma Volatiles**

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Objectives of Proposed Research
The objective of this project was to develop a sensitive, high throughput analytical method, using gas chromatography combined with tandem mass spectrometry (GC-MS/MS) to analyze 2-acetyl-1-pyrroline (2AP) in single grains of rice and plant material (e.g., leaves). 2AP is an important volatile compound associated with a desirable 'popcorn-like' aroma in aromatic rice varieties. The developed method can be used in breeding programs to readily identify rice varieties with desirable aroma characteristics.

Summary of 2013 Research (major accomplishments)
During the past year we validated a method for quantification of 2AP in rice samples. Details of the method validation are described below based on the proposed project procedures and objectives.

1) Develop GC-MS/MS conditions for optimal separation and quantification of 2AP in rice kernels and plant tissues.

   GC analysis was performed on an Agilent 7890A gas chromatography paired with an Agilent 7000B triple quadrupole mass spectrometer. The separation column was an HP-5MS UI fused-silica capillary column (30 m x 0.25 mm i.d., 1.0 μm film thickness; Agilent Technologies). Inlet and oven conditions were as follows: inlet temperature of 270°C, using a dedicated SPME inlet liner with a 0.75 mm inner diameter (Supelco, Bellefonte, PA), splitless mode after 1.2 min; constant Helium column flow of 1 mL/min; transfer line temperature of 280°C; ion source temperature of 230°C. The oven program started at 40°C, was held at that temperature for 1 min, and then was heated at 30°/min to 280°C, with a final hold of 5 min at 280°C.

   MS/MS conditions for selective identification of 2AP were identified. The multiple reaction monitoring (MRM) transitions and collision energies were optimized in order to identify ions unique to 2AP and d4-2AP, while providing optimal ion abundances for

quantification. Three MRMs (115 -> 87.1, 87, 85) have been selected for the internal standard (d4-2AP), and two MRMs (111 -> 83, 82) were identified for the analyte 2AP. The internal standard (d4-2AP) is an isotopologue of 2AP with 4 hydrogens on the acetyl side chain of the parent compound replaced with 4 deuteriums, causing a mass shift of 4 amu in the MS spectrum.

2) Optimize headspace solid phase microextraction sampling conditions (HS-SPME).
We evaluated several different SPME fiber phases for maximum response to 2AP in the GC-MS/MS. A 2 cm PDMS/DVB fiber provided best sensitivity and was used for further analyses. Optimized extraction temperature and time conditions were as follows: Samples were thermostatted for 5 min at 40°C before the SPME fiber extracted the headspace for 15 min, using a Gerstel MPS2 autosampler (Linthicum Heights, MD). Using the optimized conditions we are able to quantify 2AP in a single rice kernel (Figure 1). However, based on discussions with CA Rice Research Station collaborators, we focused our analysis on a method using 1.00 ± 0.01 g of whole rice kernels. Analyses were done prior to cooking so that we can identify the amount of 2AP that is present in the kernels initially. Future work analyzing 2AP after cooking may allow determination of the amount of 2AP that is formed as a result of thermal processing and reflecting levels consumers experience during consumption.

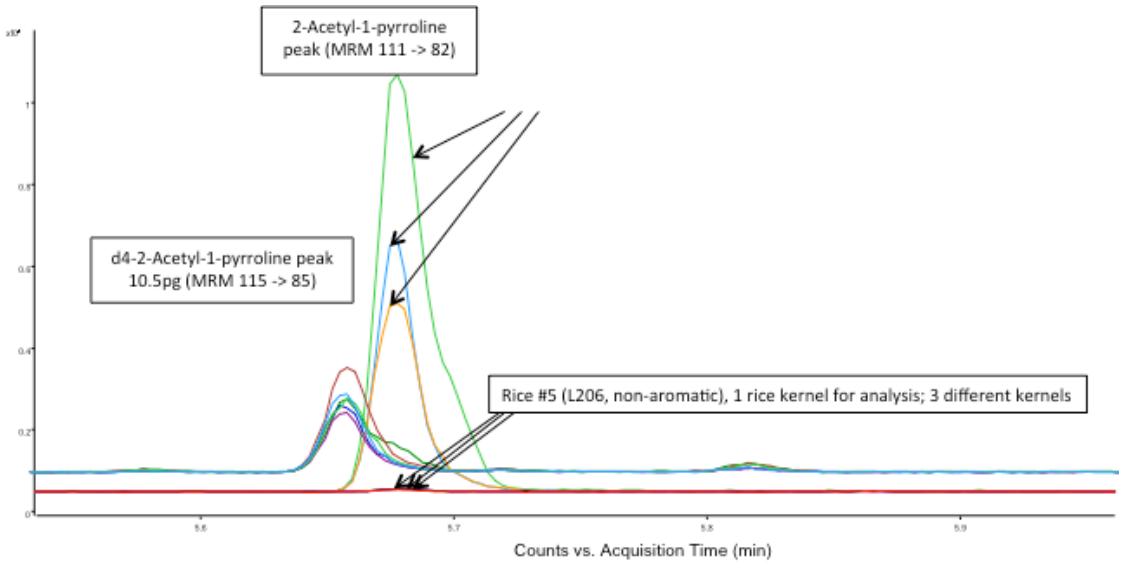

Figure 1. 2AP GC-MS peak responses in three different rice kernels from a single aromatic variety and three different kernels from a nonaromatic variety. The height (and area) of the 2AP peaks show the relative variation in the amount of 2AP among the kernels. The internal standard peak is also shown for all six samples, exhibiting good reproducibility. Peak responses have not been normalized to kernel weight (mean kernel wt for the aromatic variety is 19.3 mg; range 15.6-25.2 mg).

3) Confirm linearity, limits of quantification and limits of detection, recovery and reproducibility.

Using authentic 2AP (AromaLab AG, Freising, Germany) and a stable isotope internal standard (IS; [$^2H_4$]-2-acetyl-1-pyrroline (d4-2AP), AromaLAB AG, Freising, Germany) calibration curves containing known concentrations of 2AP spiked into nonaromatic rice varieties were generated. Calibrations were linear between the concentrations of 53 and 5380 pg/g. The limit of quantification (LOQ) was 103 pg/g with a signal-to-noise ratio of 7:1. Limit of detection (LOD) was 39 pg/g with a signal-to-noise ratio of 3:1.

Recovery and reproducibility were determined by spiking two different levels of known concentrations of 2AP into an aromatic rice variety (A-301) and extracting and analyzing using the optimized conditions determined in steps 1 and 2. Reproducibility was <10% and recoveries were between 95 and 120% (average = 109% ± 9% (s.d.), n = 4) when samples were spiked with 5.42 ng/g 2AP and between 106 and 114% (average = 112% ± 5% (s.d.), n = 4) when spiked with 538 pg/g 2AP.

4) Analyze 2AP in rice kernels.

Samples from breeding trials were provided by Dr. Farman Jodari and 2AP concentrations were determined using the validated method (Table 1). Nonaromatic varieties typically have less than 100 pg/g 2AP, and freshly milled samples (all 2013 samples were analyzed within two days after milling) have higher levels of 2AP than older samples. Concentrations in aromatic varieties are much higher than nonaromatic varieties and measured levels are generally consistent with expected breeding crosses and prior analyses obtained by the collaborators. Importantly, differences in 2AP levels were observed among samples from the same variety planted in different fields (e.g., 13-MY-37 and 13SM14181) and headrow plantings from different locations (e.g., samples 13-82364, 13-82488 and 13-82252). Some varieties/crosses also appear to be more variable within a sample (i.e., greater standard deviation for multiple analyses) than others. Grimm et al. (2001) also observed significant variability (~3-20% relative standard deviation) in 2AP levels in a SPME analysis of replicate 0.75 g samples of milled experimental rice varieties. Significant variability among metabolites between biological replicates can be common in other fruit and vegetable samples but has not been well-characterized with rice. In the upcoming year, we propose to evaluate multiple single kernels of rice within a variety to better characterize the range of 2AP levels. This may help in breeding trials to select varieties that are more consistent in their metabolite levels.

Table 1. 2AP levels in breeding samples provided by the Rice Research Station (2AP levels were determined from quadruplicate measurements. Concentration levels with standard deviation (s.d.) and relative standard deviation (r.s.d.).

Sample	2AP [pg/g]	s.d. (n=4)	r.s.d.
12 A-201	312	17.8	*6%*
12 A-301	904	120.2	*29%*
12 GH1	156	5.4	*3%*
12 GH333	362	55.5	*15%*
12 GH66	272	20.1	*7%*

12 L-206 (nonaromatic)	75	14.1	*19%*
12 SM14437	364	69.0	*17%*
12 Y1049	532	62.0	*12%*
58521	367	39.2	*11%*
MY122 (nonaromatic)	61	9.3	*15%*
MY124	234	39.9	*17%*
MY68	167	10.9	*6%*
MYP2149	178	27.5	*15%*
SJ 11-02-2011	229	7.6	*3%*
11HR57947	282	49.2	*17%*
11HR57950	378	71.7	*19%*
11HR57951	286	42.3	*15%*
11HR57952	345	31.5	*9%*
11HR57956	267	13.4	*5%*
11HR58046	286	17.1	*6%*
11HR57948	282	17.5	*6%*
MY2416	132	10.8	*7%*
11HR58324	212	26.1	*12%*
11HR58325	221	47.4	*14%*
11HR58326	200	34.7	*17%*
11HR58327	231	40.1	*17%*
11HR58451	255	46.9	*18%*
15: Y1 L-206 + Y2 A-301	191	32.8	*17%*
58323	198	23.4	*12%*
Y2 L-206 + Y2 A-301	149	11.5	*9%*
12A-301	780	93.6	*12%*
19: L-206 (nonaromatic)	68	4.8	*7%*
58521	299	70.6	*24%*
13-MY25 (nonaromatic)	66	53.5	*33%*
13-MY35	844	22.7	*13%*
13-MY37	1432	214.6	*15%*
13-MY43	1509	125.2	*8%*
13-MY90	1213	235.6	*8%*
13-MY92	852	184	*22%*
13-MY99	1165	181.9	*13%*
13-MY106	440	56.5	*13%*
13-MY107	637	12.6	*14%*
11SM14061	469	42.3	*9%*
13-82364	1914	112.9	*6%*
13-82488	1254	167.7	*13%*
13-82252	1249	327.9	*26%*
13SM14181	1013	126.7	*13%*
13SM14193	122	10.4	*8%*

Publications or Reports

We are in the process of preparing a publication based on this research. An abstract for a poster presentation has been submitted for the 2014 Rice Technical Working Group Meeting in New Orleans, Louisiana.

Concise General Summary of Current Year's Results

We have developed a rapid and sensitive method for quantifying 2AP in rice kernels. The method requires minimal sample preparation and can be used with single rice kernels or larger samples. We have applied the method to quantify 2AP in samples from breeding trials. The 2AP concentrations were generally consistent with what was expected based on the breeding crosses and on prior analyses from the Rice Station. Large variability among biological replicates was observed and potential sources of this variability will be evaluated in the upcoming year.