Comparison of amygdalin and benzaldehyde levels in California almond (*Prunus dulcis*) varietals

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Abstract

Almonds (*Prunus dulcis*), are characterized into three flavor phenotypes: bitter, semi-bitter and sweet. Amygdalin is a cyanogenic diglucoside responsible for bitterness in almonds. Studies have shown that amygdalin can hydrolyze to release benzaldehyde, which is the key component of almond aroma. In this study, the amygdalin and benzaldehyde content of fourteen sweet cultivars of almonds from four growing regions in California were determined. Solid-phase extraction and ultra high-pressure liquid chromatography coupled with electrospray triple-quadrupole mass spectrometry (UHPLC-(ESI)MS/MS) were used to determine the amygdalin content in the raw almond kernels. Headspace solid phase microextraction (HS-SPME) coupled with a gas chromatography mass spectrometry (GC/MS) was used to determine the benzaldehyde concentration in raw almond kernels. Saturated salt water was added to the sample to improve the extraction of benzaldehyde in the headspace. Results indicated that the mean concentration of both amygdalin and benzaldehyde are significantly different (*p*<0.0001) among the fourteen cultivar. Furthermore, a positive correlation was found between the amygdalin and benzaldehyde concentrations among the 14 cultivars. Although 'Nonpareil' cultivars is considered the premier snacking almond, 'Aldrich' has significantly higher concentrations of benzaldehyde in the headspace, the key contributor of the almond aroma.

Keywords: sweet almond, non-bitter almond, gas chromatography, liquid chromatography, flavor

INTRODUCTION

California (USA) is the top producer of almonds (*Prunus dulcis*) in the world, with an annual production of ~1 million tons and accounting for 80% of world almond production in 2015-2016 (Almond Board of California, 2016). Almonds are grown throughout the state of California. The main producing counties from north to south include Colusa, Stanislaus, Merced, Madera, Fresno, and Kern. The top five almond cultivars produced in California (2015-2016) are 'Nonpareil' (37%), 'Monterey' (16%), 'Butte'/Padre' (11%), and 'Carmel' (7%), followed by 'Fritz' and 'Butte' (each 6%) (Almond Board of California, 2016).

Amygdalin is a cyanogenic diglucoside that is responsible for the bitter taste in almonds (Sánchez-Pérez et al., 2008). Studies show that amygdalin is located in the kernel and is found in high levels in bitter almonds (33,006.60-53,998.30 mg kg⁻¹) in contrast to sweet almond cultivars (2.16-157.44 mg kg⁻¹) (Sánchez-Pérez et al., 2008; Lee et al., 2013). Amygdalin is degraded into glucose, benzaldehyde, and hydrogen cyanide by the activity of three enzymes after disruption of the kernel plant tissue (e.g., chewing) (Figure 1) (Sánchez-Pérez et al., 2008). The pathway of amygdalin degradation can be summed into three steps. First, amygdalin is hydrolyzed to prunasin and glucose by β-glycosidase amygdalin hydrolase (EC 3.2.1.117). In a similar manner, prunasin is hydrolyzed to mandelonitrile and glucose by β-glycosidase prunasin hydrolase (EC 3.2.1.21). Mandelonitrile can be converted into benzaldehyde and hydrogen cyanide non-enzymatically or catalyzed by the actions of mandelonitrile lyase (EC 4.1.2.10) (Sánchez-Pérez et al., 2008, 2012; Lee et al., 2013).

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Therefore, the level of amygdalin in almonds is critical for flavor, as benzaldehyde, a compound derived from amygdalin, is responsible for the almond-like flavor (Civille et al., 2010; The Good Scents Company, 2016). Benzaldehyde is an aromatic aldehyde with a pleasant almond-like aroma. This aroma compound has been found to play an important role in both bitter and sweet almonds (Wirthensohn et al., 2008; Agila and Barringer, 2012; Kwak et al., 2015). On the contrary, hydrogen cyanide is toxic and can cause both acute and sub-acute health problems at doses between 0.5 and 3.5 mg kg\(^{-1}\) body weight (Bolarinwa et al., 2014). Some symptoms include nausea, vomiting, and dizziness, and, in extreme cases, death, when the cyanide level in blood exceeds 2.6 mg L\(^{-1}\) (Geller et al., 2006; Bolarinwa et al., 2014).

Figure 1. Amygdalin hydrolysis and conversion pathway to benzaldehyde and hydrogen cyanide.

Commercial almond cultivars grown in California are all sweet. However, there is little published information on varietal differences in amygdalin and especially benzaldehyde levels, yet benzaldehyde level is critical to almond flavor. Initial analytical methods may have lacked the sensitivity to measure low concentrations of amygdalin typically found in sweet almonds (Sánchez-Pérez et al., 2008; Wirthensohn et al., 2008; Toomey et al., 2012).

More recently, an ultra-high-pressure liquid chromatography triple quadrupole MS/MS method (UHPLC-(ESI)MS/MS) was developed to improve sensitivity and to survey amygdalin levels in sweet, semi-bitter, and bitter almonds; establishing general ranges of amygdalin for phenotype identification (Lee et al., 2013; Yildirim et al., 2014). In these studies, amygdalin concentrations ranged between 2.16±1.25 to 157.44±54.01 mg kg\(^{-1}\) for sweet almonds and significant differences were observed between almonds grown in different counties (Lee et al., 2013). As a key almond aroma compound, benzaldehyde levels were measured in raw almond kernels headspace (Mexis et al., 2011; Xiao et al., 2014; Valdés et al., 2015). However, these studies focused on comparing different sample preparation treatments (e.g., roasting, coating) on the almonds instead of varietal differences. To provide a more comprehensive understanding, amygdalin and benzaldehyde levels were measured in the top 14 commercially available almond cultivars grown in California. This represents the most comprehensive sampling of almonds for amygdalin and benzaldehyde to date.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Amygdalin (>99%), benzaldehyde (>99%) and benzaldehyde-d\(_6\) (98 atom % D) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The internal standard luteolin was purchased from Indofine Chemical Company (Hilaborough, NJ, USA) and \(n\)-hexyl-\(d_{13}\) alcohol was purchased from C/D/N Isotopes Inc. (Pointe-Claire, QC, Canada). HPLC-grade acetic
acid, acetonitrile and methanol along with ACS-grade sodium chloride were obtained from Fisher Scientific (Pittsburgh, PA, USA).

Almond samples

Raw kernels of fourteen commercial cultivars of almonds were obtained from the Almond Board of California (Modesto, CA, USA). These cultivars included 'Aldrich', 'Avalon', 'Butte', 'Carmel', 'Fritz', 'Independence', 'Mission', 'Monterey', 'Nonpareil', 'Padre', 'Price', 'Sonora', 'Winters', and 'Wood Colony'. Each cultivar was collected from three different farms in four different counties (Colusa, Fresno, Kern, and Stanislaus) located in California, and represent the 2014/2015 harvest year. Two cultivars, 'Winters' and 'Avalon', had a county with only two farm samples. The almonds (0.5-1 kg per sample) were collected in oxygen barrier aluminum bags and stored at 4°C until the analysis to decrease enzyme activity and lipid oxidation. Prior to analysis, an approximate 10 g sample of almond kernels was crushed with a wooden mallet, ground by spice grinder (Waring Laboratory Equipment, Torrington, CT, USA), and sieved through a 20 mesh standard screen (WS. Tyler Industrial Group, Mentor, OH, USA).

Determination of amygdalin

The extraction method used for amygdalin from almonds was as described by Lee et al. (2013). Briefly, 50 mg of sieved almond sample was extracted in 1 mL of methanol containing 0.1% acetic acid and shaken overnight at 250 rpm. The mixture was centrifuged at 3200 × g for 15 min. The supernatant was collected and evaporated to dryness at room temperature under nitrogen gas. The sample was then reconstituted in 1 mL 0.1% acetic acid in water. A clean-up step was employed using a HyperSep C18 3 mL SPE column (Thermo Scientific, Pittsburgh, PA, USA). Amygdalin was eluted with 4 mL methanol water (40:60, v:v) and filtered through a 0.2 μm nylon filter (EMD Millipore, Billerica, MA, USA) prior to MS/MS analysis. Luteolin was used as the internal reference standard and was added to the sample after filtration at a concentration of 20 µg mL⁻¹. The amygdalin analysis was accomplished using an Agilent 1290 UHPLC system interfaced with a 6460 triple quadrupole mass spectrometer (UHPLC (ESI)MS/MS). The mass spectrometer was equipped with an electrospray ionization source (ESI) via Jet Stream Technology (Agilent Technology, Santa Clara, CA, USA). Chromatography was performed on a Zorbax Eclipse Plus C18 column (2.1×100 mm, 1.8 μm, Agilent Technologies) with the mobile phase consisting of 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B). The solvent gradient proceeded as follows: 5% B from 0-1 min, 5-20% B from 1-6 min, 20-95% B from 6-7.5 min, and hold for 2 min at 95% B. The column was then re-equilibrated at the starting condition for 1 min before the next injection. The flow rate was 0.5 mL min⁻¹ and the injection volume was 10 μL. The mass spectrometer parameters for amygdalin are described by Lee et al. (2013). Quantification of amygdalin was achieved by internal calibration curve against luteolin. The quantifier for amygdalin was the transition of m/z 456 (precursor ion) to m/z 323 (product ion), followed by two qualifiers transition m/z 179 (product ion) and m/z 119 (product ion). The transition monitored for the internal standard luteolin was m/z 285 (precursor ion) to m/z 133 (product ion).

Determination of benzaldehyde

One gram of sieved almond was weighed into a 10 mL glass headspace vial (Agilent Technology, Santa Clara, CA, USA). The internal standard was prepared by diluting n-hexyl-d13 alcohol in methanol to a final concentration of 200 µg mL⁻¹. 1 µL of internal standard was added to the almond sample, followed by 700 µL of saturated sodium chloride solution. The headspace vial was capped with a 3 mm PTFE-lined silicone septa (Supelco Co., Bellefonte, PA, USA) and vortexed for 1 min to form a paste-like mixture. The sample was incubated at room temperature for at least 15 h to establish headspace equilibrium with the least standard deviation of headspace compounds. Sample extraction and gas chromatography were accomplished using an Agilent 7890A gas chromatograph, coupled with 5975C inert XL EI/CI MSD equipped with a GC sampler 80 (Agilent Technology, Santa Clara, CA, USA). The
samples were agitated at 700 rpm and incubated at 45°C for 10 min. Headspace extraction was performed by a 1 cm 30/50 µm StableFlex DVB/CAR/PDMS fiber (Supelco Co.) at a depth of 22 mm for 40 min at 250 rpm. The fiber was then desorbed at a splitless injection at 250°C for 30 min. The purge valve was opened at 0.9 min at 50 mL min⁻¹. Helium was used as the carrier gas at a constant flow rate of 1 mL min⁻¹. Compounds were separated on a DB-Wax column (30 m × 0.25 mm, 0.25 µm, Agilent Technology). The oven temperature gradient was set at 40°C for 4 min followed with a ramp of 5°C min⁻¹ to 240°C and held for 3 min. The detector was set at EI with the source temperature at 230°C and quadrupole temperature at 150°C. The transfer line temperature was kept at 250°C. Total ion chromatograms were collected by scanning from m/z 30 to 350 with a solvent delay of 2.5 min. Identification of benzaldehyde was confirmed with authentic standards and by using the mass spectra and retention time. Quantification was accomplished by establishing an internal calibration curve with devolatized almonds using benzaldehyde-d₆. Devolatized almonds that were ground sieved almonds devolatized under vacuum (30 mm Hg) at 60°C for at least 3 days.

**Statistical analysis**

Statistical analysis was performed using XLSTAT v.2013 (Addinsoft SARL, New York, NY). Significant differences of amygdalin and benzaldehyde concentrations among almond cultivars were determined using one-way ANOVA followed by the Tukey’s HSD test. Correlation between amygdalin and benzaldehyde concentrations was tested using Pearson correlation.

**RESULTS AND DISCUSSION**

Amygdalin concentration was determined in 14 cultivars of sweet almonds grown in California (Table 1).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Amygdalin concentration (mg kg⁻¹)</th>
<th>Benzaldehyde concentration (ng g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldrich</td>
<td>76.50±23.99</td>
<td>17995.00±5886.65</td>
</tr>
<tr>
<td>Avalon</td>
<td>3.00±4.17</td>
<td>1916.34±1318.85</td>
</tr>
<tr>
<td>Butte</td>
<td>18.56±20.77</td>
<td>1869.02±1545.31</td>
</tr>
<tr>
<td>Carmel</td>
<td>26.43±14.30</td>
<td>7703.38±3394.46</td>
</tr>
<tr>
<td>Fritz</td>
<td>59.71±12.37</td>
<td>11314.96±2795.53</td>
</tr>
<tr>
<td>Independence</td>
<td>2.07±1.66</td>
<td>587.70±272.88</td>
</tr>
<tr>
<td>Mission</td>
<td>40.24±18.40</td>
<td>8806.78±4101.45</td>
</tr>
<tr>
<td>Monterey</td>
<td>46.76±15.21</td>
<td>5656.94±1845.32</td>
</tr>
<tr>
<td>Nonpareil</td>
<td>9.11±4.42</td>
<td>2768.32±1783.17</td>
</tr>
<tr>
<td>Padre</td>
<td>53.24±16.74</td>
<td>6502.74±2387.32</td>
</tr>
<tr>
<td>Price</td>
<td>1.77±1.74</td>
<td>730.61±633.26</td>
</tr>
<tr>
<td>Sonora</td>
<td>5.56±2.20</td>
<td>1132.13±433.81</td>
</tr>
<tr>
<td>Winters</td>
<td>1.62±2.10</td>
<td>892.67±821.78</td>
</tr>
<tr>
<td>Wood Colony</td>
<td>41.49±14.41</td>
<td>8654.27±2137.34</td>
</tr>
</tbody>
</table>

Statistical analysis showed that there are significant (p<0.0001) differences among the amygdalin concentration of the 14 cultivars measured (Figure 2). 'Aldrich’ (76.50±23.99 mg kg⁻¹) and ‘Fritz’ (59.71±12.37 mg kg⁻¹) demonstrated statistically significant higher levels than the other cultivars examined. This result was consistent with the results of Lee et al. (2013), which also demonstrated higher levels of amygdalin in 'Aldrich' and 'Fritz' among the sweet almond cultivars. However, our results were consistently approximately 50% lower than the previous study for all the cultivars except for ‘Butte’ (Lee et al., 2013). This may be explained by the harvest year difference between the two sets of samples. As Yildirim et al (2014) demonstrated, there were significant differences in amygdalin concentration among harvest years from the same growing region and cultivar, up to 50% difference in
concentration. Overall, the range of amygdalin measured in this study (1.62-76.50 mg kg\(^{-1}\)) is consistent with previous reported values for sweet almond cultivars (Cressey et al., 2013; Lee et al., 2013; Yildirim et al., 2014).

![Figure 2. Comparisons of amygdalin from the 14 California almond cultivars. The same grouping letters are not significantly different at \(p<0.0001\).](image)

Benzaldehyde concentrations in the headspace were significantly \((p<0.0001)\) different among the 14 cultivars measured (Table 1 and Figure 3). Levels of benzaldehyde were significantly higher in 'Aldrich' (17995.00±5886.65 ng g\(^{-1}\)) as compared to the other cultivars measured. Benzaldehyde concentration in 'Nonpareil', 'Avalon', 'Butte', 'Sonora', 'Winters', 'Price', and 'Independence' were significantly lower than the other cultivars, ranging from 587.70 to 2768.32 ng g\(^{-1}\). However, the 'Nonpareil' cultivar was not significantly different from the 'Monterey' cultivar. The observed range of benzaldehyde concentration found in raw almonds was similar to what was previously reported for commercially available sweet almonds (Mexis et al., 2011; Xiao et al., 2014; Valdés et al., 2015).

![Figure 3. Comparisons of benzaldehyde from the 14 California almond cultivars. The same grouping letters are not significantly different at \(p<0.0001\).](image)
Benzaldehyde is a hydrolysis product of amygdalin and is associated with the almond-like aroma (Sánchez-Pérez et al., 2008; Lee et al., 2013; Bolarinwa et al., 2014; Yildirim et al., 2014). As expected, a significant (p<0.0001) positive correlation (r=0.720) exists between amygdalin and benzaldehyde concentrations (Figure 4). This correlation suggests that ‘Aldrich’ and ‘Fritz’ cultivars may have more almond aroma than other sweet almond cultivars examined. As almond aroma is critical to quality, these cultivars should be considered in future breeding programs emphasizing almond aroma and flavor. The ‘Aldrich’ has a ‘Nonpareil’ seed parent and ‘Mission’ pollinizer (Lee et al., 2013). The ‘Fritz’ cultivar has ‘Mission’ and ‘Drake’ seed parents, also carrying the ‘Peerless’ lineage (Dangl et al., 2009; Lee et al., 2013). The relationship between almond tree lineage and flavor will need further investigation to conclude the effect.

Figure 4. A positive correlation relationship (r=0.720) between amygdalin and benzaldehyde concentration detected in the almond samples.

CONCLUSION

This study demonstrated the range of amygdalin and benzaldehyde concentrations among commercially available California almond cultivars. ‘Aldrich’ and ‘Fritz’ cultivars have statistically higher amygdalin and benzaldehyde levels among 14 sweet commercial almond cultivars. ‘Avalon’, ‘Independence’, ‘Price’, ‘Sonora’, and ‘Winters’ cultivars have statistically lower levels of amygdalin and benzaldehyde. This information is useful for almond breeding programs as benzaldehyde levels are a key factor in almond aroma.

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