Quantification of Amygdalin in Nonbitter, Semibitter, and Bitter Almonds (Prunus dulcis) by UHPLC-(ESI)QqQ MS/MS

Jihyun Lee, Gong Zhang, Elizabeth Wood, Cristian Rogel Castillo, and Alyson E. Mitchell

Department of Food Science and Technology, University of California—Davis, One Shields Avenue, Davis, California 95616, United States

ABSTRACT: Amygdalin is a cyanogenic diglucoside responsible for the bitterness of almonds. Almonds display three flavor phenotypes, nonbitter, semibitter, and bitter. Herein, the amygdalin content of 20 varieties of nonbitter, semibitter, and bitter almonds from four primary growing regions of California was determined using solid-phase extraction and ultrahigh-pressure liquid chromatography electrospray triple-quadrupole mass spectrometry (UHPLC-(ESI)QqQ MS/MS). The detection limit for this method is ≤0.1 ng/mL (3 times the signal-to-noise ratio) and the LOQ is 0.33 ng/mL (10 times the signal-to-noise ratio), allowing for the reliable quantitation of trace levels of amygdalin in nonbitter almonds (0.13 mg/kg almond). Results indicate that amygdalin concentrations for the three flavor phenotypes were significantly different (p < 0.001). The mean concentrations of amygdalin in nonbitter, semibitter, and bitter almonds are 63.13 ± 57.54, 992.24 ± 513.04, and 40060.34 ± 7855.26 mg/kg, respectively. Levels of amygdalin ranged from 2.16 to 157.44 mg/kg in nonbitter, from 523.50 to 1772.75 mg/kg in semibitter, and from 33006.60 to 53998.30 mg/kg in bitter almonds. These results suggest that phenotype classification may be achieved on the basis of amygdalin levels. Growing region had a statistically significant effect on the amygdalin concentration in commercial varieties (p < 0.05).

KEYWORDS: almonds, amygdalin, bitterness, flavor, LC-(ESI)MS/MS, UHPLC-(ESI)QqQ MS/MS, Prunus dulcis

INTRODUCTION

California (USA) is the top producer of almonds (Prunus dulcis) worldwide, with an estimated annual production of 1 million tons and accounting for 80% of world almond production in 2012–2013. Almonds are grown in southern (Solano, San Joaquin, Merced, Kern, Kings, Fresno, Stanislaus, Madera, and Tulare counties) and in northern California (Colusa, Solano, Tehama, Glenn, Butte, Yuba, Sutter, and Yolo counties). The top five almond varieties produced in California (2011–2012) are Nonpareil (39%), Monterey (12%), Carmel (9%), Butte (8%), and Fritz (6%). Almond kernels are sold raw or processed and are consumed as snacks (raw and roasted) in a wide variety of foods including cereals and confectionaries.

Almonds are typically characterized into three phenotypes, which include nonbitter (sweet), semibitter, and bitter. Bitterness in almonds is a monogenetic trait, and the inheritance of bitterness is recessive. In general, the bitterness of almonds may be determined by the content of the cyanogenic glycoside amygdalin. Amygdalin is a diglucoside found only in the kernels of almonds, whereas the related monoglucone, prunasin, is found in the roots and leaves and kernels of almonds. Bitter almonds contain high levels of amygdalin (3–5%) and develop a characteristic almond essence of marzipan-like aroma. Hydrogen cyanide results in a bitter perception of foods. The distinct almond essence of marzipan and almond extract is associated with amygdalin and enzymatic breakdown products (i.e., benzaldehyde and hydrogen cyanide). Hydrogen cyanide (prussic acid) can produce nausea, vomiting, and severe abdominal cramps, and at high doses it can lead to death.

Almonds are classified into categories of nonbitter, semibitter, and bitter based generally just on tasting the almond kernels (usually by the grower/breeder). Bitter almonds are easily to distinguish from nonbitter varieties; however, semibitter and nonbitter almonds are often indistinguishable. Classification of almonds based upon amygdalin levels can help breeders develop almond varieties with targeted flavor profiles (i.e., sweet, more marzipan-like, etc.).

Currently, there is little information on varietal differences in amygdalin levels in nonbitter and semibitter almonds, and analytical methods may have lacked the sensitivity to measure amygdalin at trace levels found in bitter almonds. For example, in a study of five nonbitter almond varieties of a cross between the cultivars Garrigues and Tuono, three were reported to contain no amygdalin as measured by HPLC UV−vis detection. The limit of detection (LOD) was not reported for this study. Wirthensohn et al. evaluated amygdalin in 26 nonbitter
almond genotypes (open-pollinated cross of the cultivar Mission) and reported a range between 0.00 and 87.00 mg/100 g almond with a mean value of 10.01 mg/100 g. No LOD was reported for this method. In a more recent study of amygdalin in bitter almonds, the control sample (a nonbitter almond variety) was reported to contain concentrations of amygdalin lower than LOD (200 mg/kg) as measured by ion-trap LC-MS.12

Therefore, a solid-phase extraction method was developed along with an ultrahigh-pressure liquid chromatography triple-quadrupole MS/MS method (UHPLC-(ESI)QqQ MS/MS) to improve sensitivity and selectivity. This methodology allowed for analyzing amygdalin in all types of almonds, including at the trace levels found in nonbitter almond varieties. Amygdalin levels were analyzed in 10 commercially important nonbitter almond varieties found in four common growing regions of California (i.e., Colusa, Fresno, Kern, and Stanislaus). Levels of amygdalin were also measured in semibitter and bitter almonds obtained from the University of California (UC)—Davis almond-breeding program.

### MATERIALS AND METHODS

**Chemicals and Reagents.** Amygdalin (>99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetic acid, acetonitrile, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

**Almond Samples.** Raw kernels of 10 commercial nonbitter varieties of almonds (P. dulcis) were obtained from the Almond Board of California (Modesto, CA, USA). These varieties included Aldrich, Butte, Carmel, Fritz, Mission, Nonpareil, Price, Sonora, and Wood Colony. Each variety was grown in four growing regions (i.e., Colusa, Fresno, Kern, and Stanislaus counties) in the fall of 2010. Exceptions were Butte, which was obtained from Colusa and Fresno counties, and Nonpareil, from Colusa, Fresno, and Stanislaus counties. The almonds (0.5–1 kg) were harvested, bagged, and shipped directly to UC—Davis. Raw kernels of four semibitter (i.e., 98-2-305, D1-25, p63-61, and p63-168) and six bitter almond varieties (i.e., 2100, 542_11, P. webbii, p63-169, UCD F5C-5,25, and UCD F10CD1-16) were provided by the UC—Davis breeding program. Most commercially important nonbitter almond cultivars were developed from crosses between Nonpareil and Mission varieties.

Approximately 40 g of almond kernel samples was used to make composite samples. The kernels were randomly assigned to one of two replicate groups for each sample. Each sample was crushed with a wooden mallet, ground by a mortar and pestle, and passed through a 30-mesh nylon filter prior to UHPLC-(ESI)QqQ MS/MS analysis.

**Determination of Amygdalin.** The extraction method for amygdalin was modified5 by increasing the solvent contact-shaking time and by employing a solid-phase extraction (SPE) step. Briefly, a 50 mg almond sample was extracted with 1 mL of methanol and shaken overnight (15–24 h) at room temperature at 250 rpm. The mixture was centrifuged at 4000g for 15 min. The supernatant was collected, evaporated under nitrogen gas, and reconstituted in 1 mL of 0.1% acetic acid in water. SPE is necessary to remove interfering ions that can cause ion suppression during the MS analysis, leading to lower intensity of the amygdalin peak and lower recovery. The SPE column (Hypersep C18, 500 mg/3 mL from Thermo Scientific, Waltman, MA, USA) was preconditioned with 2 mL of methanol and 2 mL of water, and the sample was loaded onto the column. An additional 1 mL of 0.1% acetic acid was used to remove remaining residues in the extraction tube. The column was flushed with 2 mL of 0.1% acetic acid in water. Amygdalin was eluted with 4 mL of aqueous methanol (methanol/water, 40:60, v/v). The extract was filtered through a 0.2 µm nylon filter prior to UHPLC-(ESI)QqQ MS/MS analysis.

**UHPLC-(ESI)QqQ MS/MS Analysis.** Amygdalin analysis was performed on an Agilent 1290 Infinity ultrahigh-pressure liquid chromatography system (UHPLC) interfaced to a 6460 triple-quadrupole mass spectrometer (QqQ MS/MS) with electrospray ionization (ESI) negative mode MS/MS.

**Figure 1.** Proposed fragmentation pathway of amygdalin in ESI negative mode MS/MS via Jet Stream Technology (Agilent Technologies, Santa Clara, CA, USA). The UHPLC was equipped with a binary pump with an integrated vacuum degasser (G1420A), an autosampler (G1426A) with thermostat (G1330B), and a thermostated column compartment (G1910C). Amygdalin was separated using a Poroshell C8, column (2.1 × 150 mm, 2.7 µm, Agilent Technologies). The mobile phase consisted of a linear gradient of 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B) as follows: 5% B, 0–3 min; 5%–20% B, 3–10 min; 20–60% B, 10–11 min; 60% B, 11–15 min. The column was re-equilibrated between injections for 5 min with initial mobile phase. The flow rate was 0.25 mL/min, and the injection volume was 10 µL.

Negative ESI mode was used. The drying gas temperatures and flow rate were 300 °C and 8.0 L/min, respectively. The sheath gas temperature and flow rate were 350 °C and 11.0 L/min, respectively. The nebulizer gas pressure, capillary voltage, fragmentor voltage, and dwell time were 45 psi, 3.5 kV, 160 V, and 200 ms, respectively. The multiple reaction monitoring (MRM) mode was utilized to analyze amygdalin. Quantification of amygdalin was achieved using an external calibration curve by measuring the area of m/z 456 (precursor ion) to m/z 323 (product ion). Two more transactions were monitored, and the respective m/z values were as follows: m/z 456 (precursor ion) to m/z 179 (product ion), and m/z 456 (precursor ion) to m/z 119 (product ion). Extracts that exceeded the linear range of the standard curve were diluted before injection.

**Statistical Analysis.** Statistical analysis was performed using IBM SPSS statistics software (v. 20.0, SPSS, Inc., Chicago, IL, USA). Significant differences of amygdalin concentrations among almond varieties and among growing region in the same variety were determined using
one-way ANOVA followed by the Duncan’s multiple-range test at $p < 0.05$. Growing region differences of Butte almonds were evaluated with an independent t test at $p < 0.05$.

RESULTS AND DISCUSSION

Breeding parents for nonbitter, semibitter, and bitter almond varieties are given in Table 1.

Amygdalin is typically extracted from almond kernels using polar extraction solvents. Herein we found that polar extraction solvents result in low recoveries of amygdalin and that recovery could be improved with the addition of acid to the extraction solvent and by employing SPE to assist in sample cleanup. Amygdalin converts to neoamygdalin (an amygdalin epimer) in aqueous solvents. Approximately 35% of the 200 ppb amygdalin standard in methanol/water, 40:60 v/v, was converted to neoamygdalin during the 24 h extraction process. This conversion could be prevented with the addition of acetic acid to the almond extract prior to SPE. Under these solvent conditions, amygdalin was stable for a week at room temperature.

A proposed fragmentation pathway for amygdalin is shown in Figure 1. MRM chromatograms indicate that amygdalin elutes at 9.6 min (Figure 2). Gradient HPLC elution improved the reproducibility and LOD of amygdalin measurement. Negative ionization resulted in better sensitivity as compared to positive ionization. UHPLC (ESI)QqQ MS/MS conditions were optimized on the major precursor ($m/z$ 456) and product ions ($m/z$ 323, 179, and 119) for amygdalin (Figure 3). Loss of the disaccharide ($m/z$ 456 → 323) resulted in the highest ion abundance (Figure 3). Transitions were also observed at $m/z$ 456 → 179 corresponding to glycosidic bond cleavage and the loss of the A1 glucose and at $m/z$ 456 → 119 corresponding to the cross-ring bond cleavage of the A1 glucose (Figures 1 and 3). Quantification of amygdalin was achieved using the MRM mode transition of 456 → 323. The transitions observed at $m/z$ 456 → 179 and 456 → 119 were used to increase analytical fidelity (qualifier transitions). Fragment ions of $m/z$ 221 and 263 were also observed at nearly equal intensities as $m/z$ 179. These ions were identified previously by Neilson et al. and correspond to the cross-ring bond cleavage of glucose A2.

The limit of detection (3 times the signal-to-noise ratio) for amygdalin is 0.1 ng/mL of extract (i.e., 0.04 mg/kg almond), corresponding to 1 pg mass on column, the LOQ is 0.33 ng/mL (0.13 mg/kg almond; 10 times the signal-to-noise ratio), and the linear dynamic range of the method is 2.5−200 ng/mL of extract with an R value of 0.998−1.000. The LOD is about 400 times lower than the lowest mean level of amygdalin observed in these samples (Table 2). Recovery was measured after the addition of two different concentrations of an

Figure 2. Negative mode ESI multiple reaction monitoring (MRM) chromatograms of amygdalin (12.5 mg/kg) in nonbitter almonds: (a) 456 → 119 (qualifier ion); (b) 456 → 179 (qualifier ion); (c) 456 → 323 (quantifier ion).

Figure 3. Negative mode product ion spectrum of amygdalin (465 $m/z$, precursor) at collision energy of 12 ev.
The amygdalin concentration varied significantly within each flavor phenotype, ranging up to a 73-fold difference in nonbitter almonds (Figure 4). The Aldrich and Fritz varieties contained significantly (*p* < 0.001) higher amygdalin concentrations (157.44 ± 54.01 and 144.87 ± 36.44 mg/kg, respectively) than other nonbitter varieties (Figure 4). Amygdalin concentrations in Butte, Price, Sonora, and Nonpareil varieties were significantly lower than in other varieties, ranging between 2.16 and 12.23 mg/kg. The Monterey, Wood Colony, Carmel, and Mission varieties had intermediate amygdalin concentrations among the 10 nonbitter almond varieties. The leading nonbitter almond variety in Spain, Marcona, contains a mean value of 30 mg amygdalin/kg, and Italian cultivars vary from 73 to 195 mg/kg. Different ratios of biosynthetic and catabolic enzymes may explain the varietal differences in amygdalin concentrations.  

![Figure 4. Comparisons of amygdalin from (a) nonbitter, (b) semibitter, and (c) bitter almond varieties. The same letters are not significantly different at *p* < 0.001.](image)

Table 2. Variety and Growing Region Comparisons of Amygdalin in 10 Commercial Varieties of Nonbitter (Sweet) Almonds

<table>
<thead>
<tr>
<th>flavor</th>
<th>variety</th>
<th>growing region</th>
<th>amygdalin* (mg/kg)</th>
<th>mean concn (mg/kg)</th>
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<tr>
<td></td>
<td></td>
<td>Colusa</td>
<td>Fresno</td>
<td>Kern</td>
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<tr>
<td>nonbitter</td>
<td>Butte</td>
<td>3.47 ± 0.17</td>
<td>0.85 ± 0.65</td>
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<td></td>
<td>Price</td>
<td>7.49 ± 0.06</td>
<td>2.49 ± 0.30</td>
<td>1.43 ± 0.05</td>
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<tr>
<td></td>
<td>Sonora</td>
<td>1.83 ± 0.18</td>
<td>7.08 ± 1.26</td>
<td>5.17 ± 0.51</td>
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<tr>
<td></td>
<td>Nonpareil</td>
<td>7.05 ± 0.56</td>
<td>12.92 ± 0.57</td>
<td>16.72 ± 1.26</td>
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<tr>
<td></td>
<td>Monterey</td>
<td>108.75 ± 1.20</td>
<td>44.87 ± 1.12</td>
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<tr>
<td></td>
<td>Wood Colony</td>
<td>78.25 ± 8.70</td>
<td>81.20 ± 3.71</td>
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<tr>
<td></td>
<td>Carmel</td>
<td>75.04 ± 5.89</td>
<td>94.72 ± 5.32</td>
<td>74.19 ± 19.52</td>
</tr>
<tr>
<td></td>
<td>Mission</td>
<td>72.47 ± 8.84</td>
<td>138.11 ± 6.06</td>
<td>68.75 ± 26.97</td>
</tr>
<tr>
<td></td>
<td>Fritz</td>
<td>133.62 ± 8.37</td>
<td>130.05 ± 3.38</td>
<td>114.91 ± 16.67</td>
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<tr>
<td></td>
<td>Aldrich</td>
<td>90.06 ± 5.01</td>
<td>214.87 ± 11.65</td>
<td>194.49 ± 1.55</td>
</tr>
</tbody>
</table>

All varieties: 63.13 ± 57.54

Mean values followed by different letters indicate significant growing region differences for the same variety at *p* < 0.05. Significant difference (*t* test) in the levels of amygdalin was noted at *p* < 0.05. *t* tests were run for Butte as only two regions were available for comparisons. ANOVAs were run on all other varieties.

Table 3. Amygdalin Levels in Semibitter and Bitter Almonds

<table>
<thead>
<tr>
<th>flavor</th>
<th>variety</th>
<th>amygdalin* (mg/kg)</th>
<th>mean concn (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>p63-61</td>
<td>523.50 ± 26.73 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D1-25</td>
<td>711.80 ± 5.66 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>98-2-305</td>
<td>960.95 ± 75.31 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p63-168</td>
<td>1772.75 ± 137.67 c</td>
</tr>
<tr>
<td></td>
<td>semibitter</td>
<td></td>
<td>992.24 ± 513.04</td>
</tr>
<tr>
<td></td>
<td>542 11</td>
<td>33006.60 ± 669.63 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2100</td>
<td>34043.65 ± 515.27 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UCD F10CD1-16</td>
<td>35026.40 ± 82.45 ab</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prunus webbii</td>
<td>39736.90 ± 3582.91 bc</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p63-169</td>
<td>44550.20 ± 3490.84 c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UCD F5C-5-25</td>
<td>53998.30 ± 1166.02 d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bitter</td>
<td></td>
<td>40060.34 ± 7855.26</td>
</tr>
</tbody>
</table>

Mean values followed by different letters are significantly different at *p* < 0.001 within each flavor phenotype.
Of the semibitter varieties tested, p63-168 contained statistically greater levels of amygdalin (1772.75 ± 137.67 mg/kg) as shown in Table 3. In general, semibitter flavor almonds contained higher concentrations of amygdalin than nonbitter flavor almonds as noted previously.4 When chewing a bitter almond, an initial slight bitterness is detected (i.e., amygdalin) within a few seconds, and a strong bitter/amaretto taste is perceived as the result of the enzymatic breakdown of amygdalin into benzaldehyde and cyanide. Therefore, both the concentration of amygdalin and enzymatic hydrolysis rates will affect the perception of bitterness.8 This may be the reason that the perception of bitterness can vary in almonds with differing levels of amygdalin. Thus, sensitive and reliable quantitative determination of amygdalin by UHPLC-(ESI)QqQMS/MS offers a more reliable measure by which to categorize almond flavor.

Bitter almond kernels contained significant concentrations of amygdalin ranging between 33006.60 and 53998.30 mg/kg as shown in Table 3. These values are similar to values, 50000 mg/kg and ~41000 mg/kg,5 reported by others. Of the bitter almond varieties tested, UCD FSC-5-25 contained the highest amount of amygdalin (53998.30 ± 1166.02 mg/kg). Although two semibitter almond varieties (p63-61 and p63-168) and two bitter almond varieties (Prunus webbii and p63-169) had the same seed parent, the amygdalin contents were significantly different (p < 0.001).

Growing region comparisons of amygdalin in the 10 nonbitter commercial almond varieties are shown in Table 2. Growing region significantly affected the amygdalin content. For example, Fritz grown in Stanislaus (200.90 ± 28.82 mg/kg) contained significantly higher amygdalin than Fritz grown in other regions (114.91–133.62 mg/kg) (p < 0.01). Also, Sonora grown in Stanislaus had significantly higher amygdalin (16.95 ± 0.37 mg/kg) than that grown in other growing regions (1.83–7.08 mg/kg) (p < 0.001). The amygdalin concentration of Monterey from Colusa was significantly higher (108.75 ± 1.20 mg/kg) than that from other growing regions (34.08–62.17 mg/kg) (p < 0.001). Mission variety from Fresno contained significantly higher amygdalin concentration (138.11 ± 6.06 mg/kg) than that from other regions (68.75–79.07 mg/kg) (p < 0.05). Aldrich grown in Fresno and Kern contained higher amygdalin (194.49–214.87 mg/kg) than Aldrich grown in Colusa (90.06 mg/kg) and Stanislaus (130.34 mg/kg) (p < 0.001).

In summary, an UHPLC-(ESI)QqQ_MS/MS method was developed for measuring amygdalin in almonds with high sensitivity (<0.1 ng/mL) and high selectivity. The Aldrich and Fritz varieties had statistically higher amygdalin concentrations among nonbitter commercial almond varieties. Amygdalin concentrations were significantly different in the nonbitter and semibitter phenotypes. Our results indicate that the combination of SPE and UHPLC-(ESI)QqQ_MS/MS can be used to reliably quantify amygdalin in nonbitter almonds and may be a useful tool for distinguishing between nonbitter and bitter varieties.

**AUTHOR INFORMATION**

**Corresponding Author**
*(A.E.M.) Phone: (530) 304-6618. Fax (530) 752-4759. E-mail: aemitchell@ucdavis.edu.*

**Author Contributions**
*J.L. and G.Z. contributed equally to this work.*

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**ABBREVIATIONS USED**
ESI, electrospray ionization; QqQ, triple quadrupole; SPE, solid phase extraction; UHPLC, ultra-high-pressure liquid chromatography

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