TECHNICAL NOTE
CRIMINALISTICS

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Cyanide and Amygdalin as Indicators of the Presence of Bitter Almonds in Imported Raw Almonds

ABSTRACT: Consumer complaints received by the U.S. Food and Drug Administration in August 2010 about raw organic almonds tasting “bitter” opened an investigation into the presence of bitter almonds in the imported product. Bitter almonds (Prunus amygdalus) contain the cyanogenic glucoside amygdalin, which hydrolyzes to produce cyanide. Ultraviolet–visible spectrophotometry was used to detect and quantitate cyanide, and liquid chromatography-mass spectrometry was utilized to detect amygdalin in the submitted samples. Control bitter almonds were found to contain 1.4 mg cyanide/g and an estimated level of 20–25 mg amygdalin/g. The questioned samples contained between 14 and 42 μg cyanide/g and were positive for the presence of amygdalin. Sweet almonds were found to be negative for both compounds, at levels of detection of 4 μg cyanide/g and 200 μg amygdalin/g.

KEYWORDS: forensic science, bitter almonds, amygdalin, cyanide, liquid chromatography-mass spectrometry, ultraviolet–visible spectrophotometry

In August 2010, several complaints were received by the U.S. Food and Drug Administration (FDA) from consumers who had purchased organic almonds from stores in Washington state. Although most of the almonds tasted “normal,” the consumers indicated that some tasted “very bitter.” The original shipment of almonds was declared as a product of Uzbekistan, a region of the world in which bitter almonds grow. This raised the possibility that wild, bitter almonds had been commingled with the sweet almonds that are typically consumed in the United States. Bitter almonds are not grown in the United States for the domestic food market (Dr. Karen Lapsley, Almond Board of California, personal communication). With respect to its goal of maintaining the integrity of the nation’s food supply, the FDA was concerned with whether this commingling was performed accidentally, as an economic adulteration, or with the intent of presenting a health hazard to the consumer.

Ripe bitter almonds contain the cyanogenic diglucoside amygdalin (Fig. 1), which produces glucose, benzaldehyde, and hydrogen cyanide under enzymatic hydrolysis by β-glucosidases (1) or by acid hydrolysis (2). Prunasin (Fig. 1), which lacks the second glucose of amygdalin, is found in unripe almonds and is converted to amygdalin during the ripening process (1,3). Amygdalin is not found in sweet almonds (1), so its detection is a specific indicator of bitter almonds. Depending on the ripeness of the almonds, a combination of amygdalin and prunasin may be present, but cyanide will be released upon hydrolysis of both compounds.

The case described by Shragg et al. (2) involves acute cyanide poisoning through the ingestion of bitter almonds. Within 15 min of consumption of 12 of the almonds, the patient experienced severe abdominal cramping and collapsed. Cyanide is highly lethal and has a rapid onset, which can lead to death before the victim obtains proper medical care (2). Because of the potential health hazard associated with the ingestion of cyanide through consumption of bitter almonds, samples of the suspect almonds were collected and submitted to the Forensic Chemistry Center for analysis. The initial evaluation of the almonds utilized spectrophotometry, a technique that is used frequently for the detection of cyanide in foods, water, and waste streams (4–8). The presence of amygdalin, the source of the cyanide, was confirmed using liquid chromatography-mass spectrometry (9).

The results presented include the detection of cyanide and amygdalin in ground composites of the submitted samples and the detection of cyanide in individual almonds. The absence of amygdalin, and therefore cyanide, in sweet almonds supports the specificity of using those compounds as indicators of the presence of bitter almonds in shipments of sweet almonds. To our knowledge, this is the first reported case of bitter almond ingestion in the United States since 1982 (2).

Materials and Methods

Samples

A locally purchased sample of raw, sweet almonds, identified as “Sweet Control,” was used for comparison purposes. A 150 g portion of the Sweet Control was ground in a food processor to make a composite.

Bitter almonds, harvested from the Arboretum at the University of California at Davis, were graciously provided by Dr. Thomas Gradziel and were identified as “Bitter Control.” A 100 g portion of the Bitter Control was ground as above.

Five separate samples of shelled, raw almonds associated with the consumer complaints were submitted to the laboratory. A single 5.8 kg portion was collected from a retail store and was identified...
Standards and Reagents

Each was ground using a mortar and pestle. Almonds in this portion were selected for individual analysis, and appearance compared to the domestic sweet almond, 20 of the almond kernel vary among cultivars. Based on the differences in Santa Cruz, CA). HPLC-grade CH3CN, HPLC-grade CH3OH, pyr-

teric acid were purchased from Sigma-Aldrich (St. Louis, MO). Aqueous standard solutions containing 0, 1, 2, 4, and 8 µg CN– were prepared by placing volumes of 0, 50, 100, 200, and 400 µL, respectively, of 20 µg/mL CN– in separate 15-mL Falcon centrifuge tubes (Fisher). An aliquot of DIH2O, equivalent to the volume of sample extract used (typically 400 µL), was also added to the tubes. The total volume was brought to 5 mL by adding an appropriate volume of 0.25 M NaOH.

The suspect samples and the Sweet Control were prepared by placing 1 g portions of each composite and 5 mL DIH2O in 15-mL Falcon tubes. The samples were shaken for 10 min and centrifuged at 10,000 × g for 10 min in a Sorvall Biofuge Primo centrifuge (Thermo Scientific, Langenselbold, Germany). The supernatant was removed and filtered through 0.45-µm Nylon filters (Fisher). Aliquots of 400 µL were transferred to separate 15-mL Falcon tubes, to which 4.60 mL 0.25 M NaOH were added.

The Bitter Control was prepared using 1 g in 5 mL DIH2O as above. After centrifugation and filtration, 20 µL of the extract was diluted further with 1.00 mL DIH2O, because of the quantity of cyanide present. A 400-µL aliquot of this diluted extract was transferred to the 15-mL Falcon tube and treated as above.

For the analysis of the individual almonds, the entire ground almond was extracted in 5 mL DIH2O as indicated. Initial experiments utilized 200-µL aliquots of the extract, mixed with 4.80 mL 0.25 M NaOH. Extracts that were found to be positive for cyanide were diluted further, 20 µL with 1.00 mL DIH2O. A 200 µL portion of this solution was mixed with 4.80 mL 0.25 M NaOH for analysis.

After the standard and sample preparations were brought to 5 mL, 1.5 mL of 1 M NaH2PO4 was added. This was followed by the addition of 200 µL of chloramine-T solution (1.0 g in 100 mL DIH2O). After 2 min, 500 µL of PBA reagent was added, followed by 2.8 mL DIH2O. The resulting solutions were centrifuged at 4500 × g for 8 min (Eppendorf Centrifuge Model 5804R; Fisher) to remove remaining particulate material, and portions were placed in cuvettes. The solutions were scanned between 500 and 650 nm using the Evolution 600 UV-Vis spectrophotometer (Thermo Scientific). Because of the background absorbance of the samples, baseline subtraction was utilized. The absorbance values at 520 and 630 nm were averaged and subtracted from the reading at 578 nm, which was used for quantitation. The limit of detection (LOD) and limit of quantitation (LOQ) were determined by making seven readings of the 1 µg cyanide standard. The standard deviation (SD) was calculated, the LOD was assigned the value (3 × SD), and the LOQ was assigned the value (10 × SD).

Ultraviolet–Visible Spectroscopy for the Quantitation of Cyanide

This colorimetric procedure is an adaptation of EPA Method 335,2, in which cyanide is converted to cyanogen chloride by reaction with chloramine-T, at a pH <8. A colored complex forms following treatment with the pyridine–barbituric acid (PBA) reagent (8).

The PBA reagent was prepared by placing 15 g barbituric acid in a 250-mL volumetric flask. Distilled water was added to wash the sides of the flask and to wet the barbituric acid. This was followed by the addition of 75 mL pyridine with mixing and the addition of 15 mL concentrated HCl. After the solution cooled to room temperature, it was diluted to volume with DIH2O and mixed.

The 1000 µg/mL CN– stock standard solution was prepared by dissolving 0.250 g KCN in 100 mL 0.10 M NaOH. The 20 µg/mL CN– working standard solution was prepared by placing a 1.00-mL aliquot of the stock CN– standard solution and 1.25 mL of 10 M NaOH in a 50-mL volumetric flask and diluting to volume with DIH2O.

Liquid Chromatography-Mass Spectrometry for the Detection of Amygdalin

Analyses were performed using a Thermo-Finnigan LTQ ion trap mass spectrometer equipped with an Ion Max source (Thermo Electron Corp., San Jose, CA) coupled to an Agilent 1100 Series HPLC system (Agilent Technologies, Santa Clara, CA). The experimental parameters were adapted from Gratz and Gamble (9). The column used was a Luna CN, 4.6 mm × 250 mm, 5-µm particle
size (Phenomenex, Torrance, CA) maintained at 30°C. An isocratic mobile phase of 98:2 (v/v) 10 mM ammonium acetate/CH3CN was used at a flow rate of 350 μL/min, and an analysis time of 20 min. The autosampler was maintained at 15°C, with an injection volume of 1 μL.

Data acquisition and analysis were accomplished using Xcalibur, version 2.0.7 (Thermo Electron Corp.). The following parameters were constant through the analyses: ionization = positive ion electro-spray; sheath gas flow = 50 arbitrary units; sweep gas flow = 15 arbitrary units; auxiliary gas flow = 15 units; spray voltage = 3.5 kV; and capillary temperature = 300°C. Five scan events for the mass spectrometry (MS) analysis were employed. The first was to collect MS data over the range m/z 473–477 for amygdalin. The second event collected the tandem mass spectrometry (MS/MS) spectra over the range m/z 130–500 for m/z 475, which is the [M+NH4]+ ion for amygdalin, and the third collected MS3 spectra over the range m/z 85–350 on the transition from m/z 475 → 325 for amygdalin. The fourth event collected MS data over the range m/z 311–315 for prunasin, and the fifth collected MS/MS spectra over the range m/z 85–350 for m/z 313, which is the [M+NH4]+ ion for prunasin. The second, third, and fifth events utilized a normalized collision energy of 30% using helium as the collision gas.

Standards containing between 5 and 25 μg/mL amygdalin, and a standard containing 10 μg/mL prunasin, were prepared in DIH2O. For sample analysis, a 1 g portion of each composite was extracted in 25 mL CH3OH and sonicated for 15 min. An aliquot of the extract was filtered through a 0.2-μm PTFE syringe filter (Fisher) and further diluted, 100 μL with 900 μL DIH2O, prior to analysis. Because of the higher level of amygdalin in the Bitter Control, the filtrate was diluted further, 10 μL with 990 μL DIH2O. LOD experiments were conducted by preparing a series of amygdalin fortifications of the Sweet Control. The estimated LOD was the lowest level of fortification at which the amygdalin ions were observed.

Results

Quantitation of Cyanide

To determine whether the consumer complaint samples contained bitter almonds, aqueous extracts of the Sample and Control composites were prepared. The spectrum of each treated solution was recorded from 500 to 650 nm. Figure 2 shows the spectra collected for the blank, the Sweet Control, the Bitter Control, and Sample 1 composite 3. The responses for the blank and the Sweet Control were the same, indicating the lack of cyanide in sweet almonds. Both Sample 1 composite 3 and the Bitter Control gave positive responses for the presence of cyanide. Background absorbance from the matrix can also be observed in the responses from Sample 1 composite 3 and the Bitter Control, necessitating baseline subtraction prior to quantitation. As summarized in Table 1, the distribution of cyanide throughout the lot varies somewhat, suggesting that the source of contamination is not distributed uniformly throughout the shipment. However, it is also possible that some of the variance among samples is attributable to sampling issues within each composite or to incomplete hydrolysis. The LOD and LOQ were determined to be 4 and 13 μg/g cyanide, respectively. Although no attempt was made to optimize the extraction protocol or to ensure complete hydrolysis, it can be estimated that Samples 1 through 5 contained between 0.1% and 1.7% (w/w) bitter almonds. For shelled almonds, the U.S. Department of Agriculture permits up to 1% bitter almonds mixed with sweet almonds (11). There are no established limits for permitted quantities of cyanide that can be ingested through foods.

Additional experiments were conducted on individual almonds, in an attempt to identify the bitter type. Twenty were selected from Sample 1 based on their appearances, and 18 of those were negative for cyanide. Two of the almonds contained 2.1 mg and 2.8 mg CN−/g, or 2.2 mg and 3.6 mg CN− per almond, respectively. These values are lower than those reported by Shragg et al. (2) at 6.2 mg per almond, with a range of 4–9 mg per almond. However, they are similar to the 290 mg HCN per 100 g of tissue reported by Micklander et al. (12). These data are not meant to suggest that Sample 1 contained 10% bitter almonds, but merely to indicate the ability to confirm their presence.

Detection of Amygdalin

As shown in Figure 3, amygdalin has a retention time of 13.2 min. The selected-ion monitoring (SIM) MS spectrum has a peak at m/z 475, which is the [M+NH4]+ ion of amygdalin. Previous work indicated that the use of ammonium acetate in the mobile phase promoted the formation of the ammonium adduct, which greatly enhanced the signal-to-noise ratio over that obtained from the protonated species (9). In the MS/MS spectrum, there are ions at m/z 458, 325, and 163. The ion at m/z 458 represents the

![Absorbance Spectra Comparison](https://via.placeholder.com/150)

FIG. 2—Comparison of absorbance spectra obtained from method blank (○), Sweet Control (×), Bitter Control (Δ) and Sample 1 composite 3 (□).
[M+H]+ ion of amygdalin. The other ions observed represent loss of water from the ammonium adducts of the diglucoside and monoglucoside, respectively. The MS3 (475 → 325) spectrum contains ions at m/z 289, 271, 163, 145, and 127.

Because it was not known whether the imported almonds were fully ripened, it was not known whether the source of the cyanide could have been prunasin. Prunasin has a retention time of 15.1 min. The SIM MS spectrum for prunasin, which has a molecular weight of 295, contains a peak at m/z 313, corresponding to the [M+NH4]+ ion. In the MS/MS spectrum, there are ions at m/z 296, 278, 180, 163 and 145 (data not shown).

Figure 4 shows the amygdalin MS3 data obtained from the two control samples and one of the suspect samples. By monitoring the 475 → 325 transition, it is possible to eliminate any potential false positives that may occur from matrix components having a m/z of 475. Amygdalin was identified in Samples 1 through 5, and in the Bitter Control, based on retention time and MS comparisons to the amygdalin standard. Based on peak areas obtained from the extracted ion chromatogram for m/z 163 in the MS3 data, it was estimated that the Bitter Control contained between 20 and 25 mg/g amygdalin. This corresponds to 1.1–1.4 mg/g cyanide, which correlates with the values obtained using the spectroscopic method. As is shown in Fig. 4, amygdalin was not detected in the Sweet Control, with an estimated LOD of 200 μg/g. Prunasin was not detected in any of the samples.

Micklander et al. (12) report an amygdalin value of 110 nmol/mg in bitter almonds, or 50 mg/g. According to Sánchez-Pérez et al. (1), amygdalin is detected in the bitter almond genotype “S3067,” the slightly bitter genotype “Garrigues,” and the sweet heterozygous genotype “Marcona,” but not in the sweet homozygous genotype “Ramillete.” The levels detected in the Garrigues and Marcona genotypes were 0.03 and 0.007 μmol/100 mg, respectively (1), which are equivalent to 0.13 mg and 0.03 mg/g. Amygdalin content in the bitter almond genotype “S3067” was 9 μmol/100 mg (1), or 41 mg amygdalin/g. When this value is converted to the equivalent amount of cyanide, it represents 2.5 mg CN⁻/g.

Conclusions

The detection of cyanide and amygdalin in samples of imported almonds served as indicators of the presence of bitter almonds that had been commingled in the product. Unfortunately, because it is not known which species of almonds are present in the imported sample, direct comparisons of amygdalin and cyanide levels to specific genotypes are not possible. Hence it is not possible to ascertain the actual levels of adulteration, which may be used to indicate attempted economic fraud. From a food safety perspective, a minimum lethal dose of cyanide is reported as 0.5 mg/kg, or 50 mg for a 100 kg (220 lb) adult (2), while an average lethal dose is 1.4 mg/kg (13), or 140 mg for a 100 kg adult. At a level of 3.6 mg cyanide/almond from this shipment, a consumer would need to ingest more than 40 of the bitter almonds. In a health hazard assessment conducted by the FDA’s Center for Food Safety

| Sample                | Cyanide, μg/g | LOD*  |
|-----------------------|--------------|-------|
| Sweet control         | < LOD*       |       |
| Bitter control        | 1400         |       |
| Sample 1, composite 1 | 18           |       |
| Sample 1, composite 2 | 40           |       |
| Sample 1, composite 3 | 23           |       |
| Sample 2              | 14           |       |
| Sample 3              | 42           |       |
| Sample 4              | 24           |       |
| Sample 5              | 42           |       |

LOD, limit of detection.

*LOD = 4 μg cyanide/g almond.
and Applied Nutrition (13), it was concluded that, although the levels of cyanide found were of concern, it was unlikely that the consumer would ingest the large number of almonds needed to present an immediate health risk. No actions were taken. Further analyses are being conducted to optimize the extraction procedures for both cyanide and amygdalin, to ensure quantitative determination of the levels present and to assess accurately any potential health hazard that the consumer may face.

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