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Attempted Quantification of the Cyanogenic Glycosides Prunasin and Sambunigrin in the Sambucus L. (Elderberry)

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ATTEMPTED QUANTIFICATION OF THE CYANOGENIC GLYCOSIDES PRUNASIN AND SAMBUNIGRIN IN THE *SAMBUCUS* L. (ELDERBERRY)

by

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Abstract

Food and health industries are taking advantage of the phenolics in elderberries (*Sambucus* L.) to relieve symptoms of ailments like the flu. A rise in demand has induced an increase in the production of elderberry products. Although the pharmacological attributes of these fruits have been investigated, the toxicology has not been well addressed. While inedible species such as *S. glauca* have been found to contain cyanogenic glycosides, there is not a clear understanding of the toxicity of these plants. These cyanogenic glycosides hydrolyze to form the toxin hydrogen cyanide (HCN) when consumed, causing the clinical symptoms of nausea, vomiting, headache, dizziness, cyanosis, liver damage, hypotension, fever, mental confusion, and even death. For this reason, the consumption of these fruits in the raw state may pose a hazard to humans. Before consumption it is recommended that berries be de-stemmed, seeded, and cooked to avoid toxic effects.

The purpose of this study was to quantify the cyanogenic glycosides prunasin and sambunigrin in elderberries native to Maine. This research observed that prunasin standard chemically changes over time while in a methanol solution. This change may be from degradation or from reactions with unknown impurities in the standard. This study also found that standard can be removed from methanol and later re-dissolved to prevent this chemical change, which may save time and money in future studies. Another finding in this study is that the best HPLC-UV chromatographic separation occurs in an acid-free mobile phase. Finally this study found that an ELSD should be used to quantify prunasin.

Acknowledgment

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Table of Contents

List of Figures

List of Tables

1. Introduction

The elderberry is a member of the *Adoxaceae* family, sometimes referred to as the *Caprifoliaceae* or *Sambucaceae* family, and is of the genus *Sambucus* L. One of the most prevalent species is the American elder, referred to as *Sambucus nigra,* subsp. *canadensis* [1]. Some of the common American elder cultivars include Bob Gordon, Dallas, Ocoee, Ozark, and York[1].

First seen in Europe and Africa, the elder plant is now found in Asia, North America, New Zealand and Australia[2]. The bushes grow best in fertile, water-rich terrains with moderate temperatures and that are free from heavy rain and winds[2]. Some of the common places that cultivate the elder plant are the United States (mainly the northern and Midwest portions of the country), Ukraine, Denmark, Austria, The Netherlands, Scotland, Germany, Japan, Russia, and Great Britain[3]. The phenolics, sugars, and minerals of the elder plant vary among plant parts and cultivars, as well as among the same genotype depending on location and time of growth, soil composition, water levels, and pests[1, 2, 4].

Although the compositions vary widely, the morphology of elderberries does not. The berries are either ovate or slightly oblong with three to five berries per node[3]. The berry color has been found to be blue-black, deep red, and bright red-orange[3], Figures 1 and 2. The flowers of the fruit vary the least, as they are always a white or cream color[3], Figure 3. Finally, the berries are grown on either a tree or shrub[3], Figures 4 and 5.

Figure 1: A deep-red colored elderberry, common to *Sambucus nigra,* subsp. *canadensis*. (Reproduced from reference [5]).

Figure 2: A blue-black colored elderberry, common to *Sambucus nigra,* subsp. *nigra.* (Reproduced from reference [6]).

Figure 3: The white, creamy elderflower characteristic of all elderberries. (Reproduced from reference [7]).

Figure 4: The elder tree. (Reproduced from reference [8]).

Figure 5: The elder shrub. (Reproduced from reference [9]).

Although the literature on elderberry marketing is limited, elder plant commerce has increased in the last ten years, paralleled with new information on the nutritional characteristics of the plant[10]. A survey of people involved in the elderberry market in Missouri found that 59% of respondents will have an increasing demand and 19% will have a steady demand for elderberry products[10]. The marketing of elderberry products is expected to continue increasing with the high demand[11]. Moreover, although the establishment of an elder farm is more expensive than other crops, about $3,500 per acre, the estimated rate of return on the investment can be up to 15%, encouraging farmers to implement these crops[12].

Historically, the elder tree was used to treat illnesses such as peritonsillar abscesses, sore throats, rheumatism, warts, epilepsy, and pneumonia[13]. Prior to scientific understanding of the plant, the elder tree was considered to heal with its energy as the

elder tree was thought to absorb illness and negative energy, either through direct contact or within proximity[13]. Many medicinal instruments included elder tree ornaments such as amulets to cure rheumatism[13]. To cure epilepsy it was believed that the affected individual could simply lie under an elder tree after the first seizure[13]. More direct treatments were made from the berries and flowers in the form of syrups, tinctures, oils, ointments, liniments, salts, vinegar, sugars, teas, and wines[13].

In the modern world, elderberries are still used to manage many ailments such as constipation, influenza, upper respiratory tract infections, arteriosclerosis, type-2 diabetes, hypertension, dementia, and is even thought to help prevent or treat cancer[14]. Elder flowers are sold in Ukraine and Russia to relieve congestion, fever, and inflammation. Sinupret®, a combination of elderflowers and other botanicals, has been approved by the European Union to alleviate symptoms of the common cold[14]. Elderberries have been shown to be hepatoprotective and gastroprotective against toxins[14]. These widespread medicinal uses of the elderberry are attributed to the antioxidant, anti-inflammatory, antiproliferative, analgesic, and immunomodulatory effects[14]. High levels of phytochemicals, also found in other plants such as chokeberries and blueberries[14], are thought to be responsible for these beneficial properties. Lectins in elderberries have also been shown to promote DNA damage repair by helping to up-regulate the human DNA repair enzyme methylguanine methyltransferase (MGMT). These lectins are also known to support the immune system by increasing antibody production, which can help prevent illness. Finally, lectins have been demonstrated to stimulate insulin release to lower blood glucose levels, which may help to manage type-2 diabetes[15, 16].

Other than their medicinal purposes, elderberries are used, both historically and modernly, to make dyes, jams, sauces, flavoring, yogurt, ice cream, and beverages[2, 17]. Traditionally in Spain, Germany, Romania, Sweden, and France elderflowers have been used to make cordials[15]. Due to their popularity, some commercial companies have started producing elderflower-flavored drinks such as Fanta Shokata[15].

Although great research has been done on the health benefits of the elder plant, very little has been done on its toxicology. There have been reports of relatively minor elderberry poisoning with no lethal events. For example, in 1983, eight people presented to a hospital with acute gastrointestinal and neurologic symptoms after consuming a juice created by a religious center that had been prepared with elderberries, branches, and leaves[18]. Eleven others who had consumed less of the juice complained of nausea, dizziness, numbness, abdominal cramps, and weakness. Further investigation found that severity of symptoms was positively correlated to the amount of juice consumed[18]. These incidents were likely caused by cyanogenic glycosides.

Cyanogenic glycosides are naturally occurring compounds that can be metabolized to release the toxin cyanide. In the 1970s, Seigler and coworkers investigated the structures of twenty-nine naturally occurring cyanogenic compounds from a variety of plants such as the Mexican Buckeye and almond[21]. Derived from amino acids and sugars, these compounds help aid in host defense as a toxin to predators[21, 22]. Cyanogenic glycosides have been found in the seeds, skins, and leaves of over 2500 plant species including apples, apricots, plums, bamboo shoots, legumes, flax seeds, and elderberries [20, 21]. Although these cyanogenic glycosides are not hazardous in their natural state, they release toxic hydrogen cyanide (HCN) when metabolized. Natural

cyanogenic compounds have been the cause for accidental, and intentional, poisoning for many years as they have been consumed unknowingly by animals and humans alike [22, 23]. Cyanogenic glycosides in elderberries are prunasin, sambunigrin, zierin, and holocalin[17, 19, 20], Figure 6.

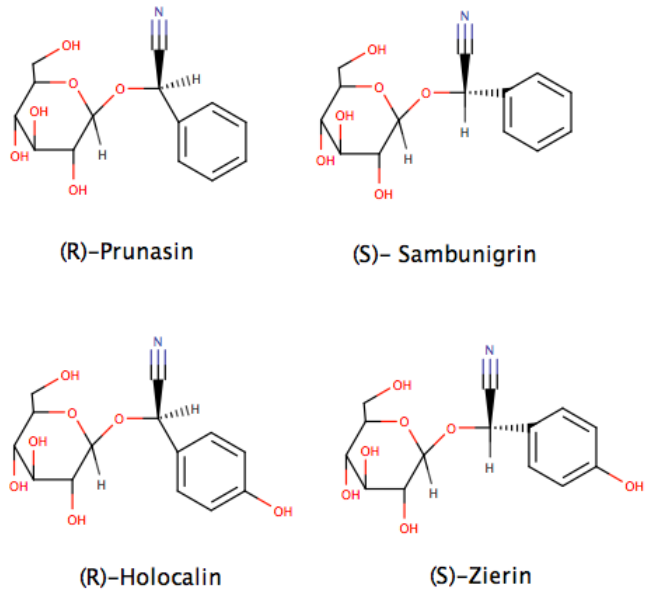


Figure 6: Structures of the four cyanogenic glycosides found in the elderberry. (Reproduced from Seigler [21])

When plants are disturbed, through chewing or crushing, their cyanogenic glycosides interact with water and endogenous β–glucosidase and α–hydroxynitrile lyase, releasing HCN until the enzyme is denatured by the low pH of the stomach[24, 25], Figure 7. This process is enhanced in the lower gastrointestinal tract as exogenous β–glucosidase of the bacteria in the intestines catalyzes the formation of HCN [24-27]. After absorption in the intestines, the toxin binds to erythrocytes in blood as the CN^- binds to the iron in both hemoglobin and methemoglobin[24]. As it travels through the circulatory system, CN^- is absorbed by cells throughout the body where it inhibits

oxidative phosphorylation, a process essential to cell metabolism, by binding with the hemes of enzymes. This prevents oxygen from being used as a final electron acceptor, also known as histotoxic anoxia [24, 26, 28]. This process causes the clinical symptoms of nausea, vomiting, headache, dizziness, cyanosis, liver damage, hypotension, fever, mental confusion, and even death [26].

The human body can tolerate low levels of HCN because of the conversion of HCN into thiocyanate by the enzyme rhodanese, which is then excreted in urine[24]. The acute toxic oral dose of HCN for humans is reported to be between 0.5-3.5 mg/kg body weight while the lethal dose is 60 mg/kg body weight[24]. These values, however, are difficult to determine, as this experiment cannot ethically be performed on humans. Thus, these values come from examination after death. Plants releasing more than 20 mg HCN/ 100 g of fresh plant material are considered dangerous to consume. Other factors that determine whether cyanide poisoning will occur include the body weight of the consumer, amount of cyanogenic glycoside present in the plant, and the speed of hydrolysis[19]. This is important because more factors than the presence of a compound with the ability to release cyanide contribute to poisoning, which is of relevance to humans.

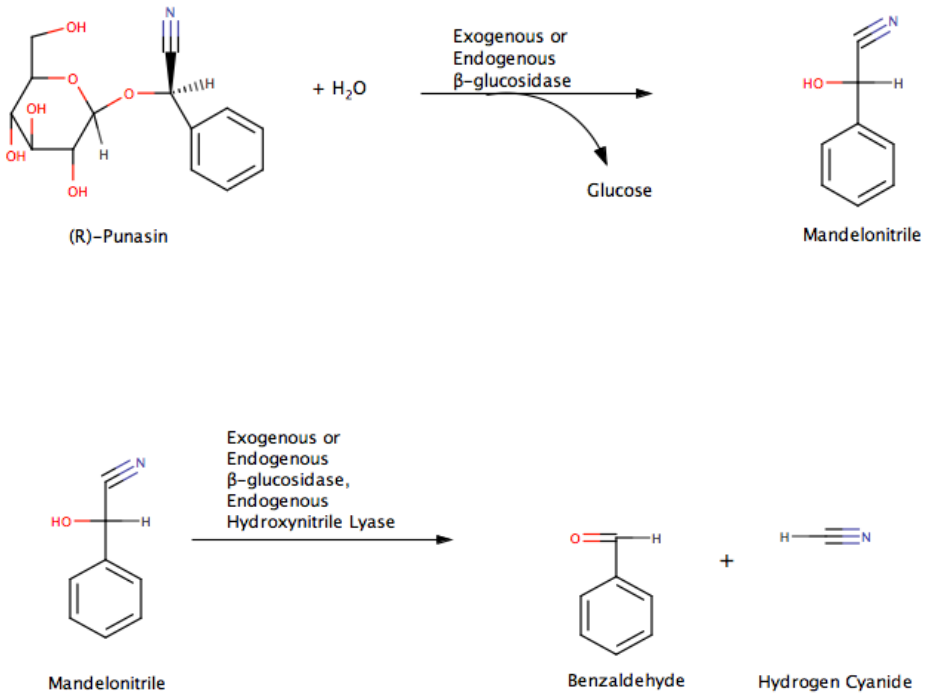


Figure 7: Reaction mechanism of the production of hydrogen cyanide from the cyanogenic glycoside prunasin in elderberries. The diastereomer, sambunigrin, performs similarly. (Scheme generated from references [21, 22, 25, 29].)

The concentration of cyanogenic glycosides differs among the different components of a plant. For example, some plants contain a higher concentration of cyanogenic compounds in the leaves and stems, while the content is higher in the seeds/kernels of others[30]. This is a concern to the elderberry industry as many of the products made today are juices where the possibility of cyanide contamination from seeds and small stems is possible. Industry uses heat to treat products, which helps to catalyze the hydrolysis of prunasin to hydrogen cyanide gas. This gas is then released into the atmosphere, thus is not found in the products that consumers receive.

As the toxin HCN is of interest due to its biological implications, qualitative studies have shown the presence of HCN released through the use of picric acid paper[22, 30-32]. Buhrmester and coworkers found a range of released HCN to be between 0 and greater than 50 mg HCN per kg fresh weight elderberries native to Illinois[30]. This broad range of released HCN shows the imprecision of picric acid. This variation is mainly due to how difficult it is to retain all HCN that is released as some is lost in the preparation of the sample. Thus this method is used more as a qualitative measure[33]. This test also does not quantify the cyanogenic glycosides in a sample, as only the HCN released is measured. Although research has shown that about 80% of HCN that a body is exposed to will be excreted as thiocyanate and the remaining 20% will have toxic effects, little is known about what happens to remaining cyanogenic glycosides in the body that are not hydrolyzed to HCN[24]. Thus, quantifying the cyanogenic glycosides in a sample may provide more information on the effects of consuming the berries as opposed to only measuring the HCN released.

Other methods of quantification exist that have better accuracy and precision than picric acid paper. High Performance Liquid Chromatography (HPLC) and Ultra Performance Liquid Chromatography (UPLC) are used in a variety of industries and are accurate methods of quantitative analysis. Although HPLC and UPLC have been used to quantify cyanogenic glycosides in other plants like flax [34], apples[35], and clovers [35], they have not been used for the analysis of elderberries.

With the increasing demand for elderberry products, more information is needed on the toxicology. The objective of this study was to develop a method and quantify the cyanogenic glycosides, prunasin and sambunigrin, in elderberries from Maine. Due to the

unavailability of holocalin and zierin standards, their method development and quantification was not feasible.

2. Experimental Methods

2.1 Elder Plant Collection and Storage

The new and old stems, leaves, and berries of elder plants were collected from the UMaine campus garden in October of 2015 and separated by stripping off the berries and leaves followed by breaking the new stems off of the old stems. Old stems were defined as those that were woody while new stems were those that were not woody. The stems, leaves, and berries were stored separately at -20˚C.

2.2 Seed Separation

Wild Maine elderberries were harvested in Penobscot County in September of 2014 and stored at -20˚C until use. The berries were allowed to thaw at room temperature and were lightly crushed using a mortar and pestle to separate the seeds from the flesh and skin. Approximately 7 g of the crushed berries were put in a 1.0 L beaker with 750.0 mL of deionized water, and the beaker contents were stirred vigorously for ten minutes. The contents were allowed to rest until the majority of the seeds settled, about two minutes. Skins at the top of the beaker were decanted, and the volume was returned to 750.0 mL with deionized water. The stirring-resting-decanting procedure was repeated three more times, and the resulting seeds, skins, and flesh were filtered using a 40 μm sieve. After being washed with deionized water, the skins remaining with the seeds were manually removed with tweezers. This separation process was done on the remaining crushed berries. The seeds were then sandwiched between two Whatman No. 1 filter papers and allowed to dry at room temperature overnight. The next day the seeds were scraped from the filter papers, leaving any remaining bits of flesh behind. The seeds were then stored at -20˚C.

2.3 Elder Leaf and Stem Freeze Drying

The manually separated leaves and old and new stems as described above were dried for 72-hours in a VirTis Ultra freeze drier with the program Encore (SP Scientific, Gardiner, NY, USA).

2.4 Solvents, Chemicals, and Standards

High Performance Liquid Chromatography (HPLC) grade methanol and acetonitrile were purchased from Fisher Scientific (Pittsburg, PA, USA). Formic and acetic acid, ferrous sulfate, hydrochloric acid, and sodium hydroxide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fluka brand (Sigma-Aldrich, St. Louis, MO, USA) trifluoroacetic acid also was used.

Prunasin standard was obtained from Sigma-Aldrich (St. Lois, MO, USA). A 1.0 mg/mL stock solution was prepared using HPLC grade methanol and was stored at -20˚C. A second prunasin standard was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Stock solutions of 3.0 mg/mL and 0.5 mg/mL were prepared using HPLC grade methanol and were stored at -20˚C.

2.5 Qualitative Prussian Blue Test

A qualitative Prussian blue test was developed based on that described by Lee-Jones[36, 37] using apple seeds, which are known to contain the cyanogenic glycoside amygdalin. This method was then used qualitatively to test the previously separated wild Maine elderberry seeds for cyanogenic glycosides. These elderberry seeds were crushed using a mortar and pestle and weighed using an analytical balance. Weighing was done after crushing as some seed mass was lost in the crushing process. A 0.05:1 ratio of

crushed elderberry seeds to 5.0% sodium hydroxide (by volume) were stirred on a stir plate for thirty minutes. The mixture was then filtered using Whatman No. 1 filter paper, followed by a wash with 5.0% sodium hydroxide. This filtrate was then heated at 40˚C on a stir plate set at 700 RPM for two minutes after adding 9.00 mL of ferrous sulfate. The mixture was then removed from heat, and ten drops of concentrated hydrochloric acid were added dropwise. The formation of a light blue/green precipitant indicated the presence of cyanide in the sample, a positive result for cyanogenic glycosides.

2.6 Instrumentation and Solvent Systems

High Performance Liquid Chromatography with ultraviolet detection (HPLC-UV) and electrospray ionization mass spectrometry detection (HPLC-ESI/MSD) were used in this study. Both positive and negative (ESI^+ and ESI^-) modes were used.

Chromatographic separation was performed on a Kintex C_{18} HPLC column (5 µm, 4.6 x 250 mm, Phenomenex, Torrance, CA, USA) equipped with a matching Kintex C_{18} guard column. Both columns were held at 25˚C. An auto-injector injected 20 µL of sample. HPLC-UV analysis was done on an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) with a diode array detector (DAD). The DAD detector allowed for the analysis of samples at multiple wavelengths simultaneously. By using the DAD, prunasin was found to have the highest absorption at a wavelength of 200 nm after testing the absorbance at 200 nm, 280 nm, and 320 nm. Literature[39] provided two maximum absorbance peaks for prunasin, 200 nm and 280 nm, thus those wavelengths were examined. Presence of solvents in these UV chromatograms was analyzed by measuring absorption at 320 nm, where prunasin is known to not absorb[39].

When mass spectrometry was performed, the HPLC-UV system was connected to an Agilent LC-MSD Trap. The MS conditions were as follows; drying gas flow rate 9.0 L/min, gas temperature 350˚C, pressure of nebulizer gas 50 psi, compound stability 100%, threshold 100,000 for ESI^- and ESI^+, scan range 50-400 amu, and the amplitude voltage used for fragmentation was 1.0 V. Although ESI^- was used predominantly for mass spectral analysis, ESI^+ was tested. Data was collected using Agilent chemstation software (Agilent Technologies, Palo Alto, CA, USA).

The solvent systems used varied throughout the study, Table 1. When 0.4 mL/min and 0.2 mL/min flow rates were used, for all mobile phases, a gradient profile of (0:10%, 30:30%, 50:10%) was used, but when a 1.0 mL/min flow rate was used the gradient profile was (0:10%, 20:30%, 30:10%). These gradients were initially selected based on previous work conducted in our laboratory. With multiple variables to test, the gradient was not changed due to time constraints. Most research on cyanogenic glycosides used isocratic solvent systems; however this research predated the use of gradient systems. Since gradient solvent systems have more recently been shown to better separate samples, a gradient solvent system was chosen for this study.

Table 1: Solvent systems used in HPLC-UV and ESI/MS analysis. Solvent systems A-F were used when HPLC-UV and ESI/MS analysis were conducted simultaneously while system G was used when only HPLC-UV analysis was done.

Solvent System	Inorganic Solvent	Organic Solvent	Flow Rate (mL/min)
A	Ultrapure water with 0.5% formic acid	Acetonitrile with 0.5% formic acid	0.4
B	Ultrapure water with 0.5% formic acid	Acetonitrile with 0.5% formic acid	0.2
C	Ultrapure water with 0.01% trifluoroacetic acid	Acetonitrile with 0.01% trifluoroacetic acid	0.4
D	Ultrapure water with 0.1% trifluoroacetic acid	Acetonitrile with 0.1% trifluoroacetic acid	0.4
E	Ultrapure water with 0.05% trifluoroacetic acid	Acetonitrile with 0.05% trifluoroacetic acid	0.4
F	Ultrapure water with 0.02% acetic acid	Acetonitrile with 0.02% acetic acid	0.4
G	Ultrapure water	Acetonitrile	1.0

2.7 Cyanogenic Glycoside Extraction

Four cyanogenic glycoside extraction protocols were tested on the berries, seeds, stems, and leaves of elder samples, as described below:

I. Seed, stem, and leaf samples were crushed, separately, in a liquid nitrogen-chilled mortar and pestle and added to a round bottom flask with ice-chilled 80.0% methanol. The mixture was refluxed vigorously for eighty minutes at 60˚C with boiling chips, followed by vacuum filtration with Whatman No. 1 filter paper. The filtrate was dried by rotary evaporation (Büchi, New Castle, DE, USA) at 60˚C. If possible, the dried extract was poured into a vial for storage, however if needed, 5.0 mL of 80.0% methanol was added to remove the extract from the flask. The extract was stored at -20˚C until HPLC analysis. The extract was filtered using a 0.45 μm syringe filter immediately before HPLC analysis.

II. Seed samples were crushed in a liquid nitrogen-chilled mortar and pestle and added to a round bottom flask with ice-chilled 80.0% methanol. The mixture was sonicated (Fisher Scientific FS30, Waltham, MA, USA) for 30 minutes followed by vacuum filtration with Whatman No. 1 filter paper. The filtrate was dried overnight in an uncovered flask (to allow for evaporation) at 2˚C. The extract was stored at -20˚C until HPLC analysis. The extract was filtered using a 0.45 µm syringe filter immediately before HPLC analysis.

III. Seed samples were crushed in a liquid nitrogen-chilled mortar and pestle and added to a round bottom flask with ice-chilled 80.0% methanol. The mixture was stirred for five hours at room temperature, followed by vacuum filtration with Whatman No. 1 filter paper. The filtrate was dried overnight in an uncovered flask (to allow for evaporation) at room temperature. The extract was then stored at -20˚C until HPLC analysis. The extract was filtered using a 0.45 µm syringe filter immediately before HPLC analysis.

IV. Whole berries were crushed in a liquid nitrogen-chilled mortar and pestle and added to a round bottom flask with ice-chilled HPLC grade methanol (99.9%). The mixture was refluxed vigorously for eighty minutes at 60˚C with boiling chips, followed by vacuum filtration with Whatman No. 1 filter paper. The filtrate was dried by evaporation on a hot plate in a fume hood. Methanol, 0.5 mL, was added, and was immediately filtered using a 0.45 µm syringe filter and analyzed using HLPC.

2.8 Investigation of Santa Cruz Biotechnology Standard

Impurities observed in the prunasin standard were analyzed two ways. First, mass spectrometry fragmentation (MS/MS) using solvent system B, Table 1, was performed to analyze an unknown compound. Second, mass spectra of prunasin standard with solvent system B were compared to that using solvent system F, Table 1, to examine the standard in different mobile phases to help determine the source and identity of the impurities.

Changes in the prunasin stock solution were analyzed over 48 hours. The 0.3 mg/mL prunasin standard was dried by running nitrogen gas over the solution to evaporate the methanol and was stored at -20˚C for approximately 60 hours. The dried standard was then re-dissolved in HPLC grade methanol to a concentration of 0.3 mg/mL and analyzed with HPLC. The Sigma-Aldrich standard was not examined due to the chemical change that all stock solutions experienced.

Results and Discussion

3.1 Qualitative Determination of Cyanide

As originally described by Lee-Jones[37,38], the presence of cyanide in a sample can be confirmed or denied by providing the conditions for the formation of the Prussian blue complex. This test required dissolving the sample in sodium hydroxide, followed by the addition of ferrous sulfate. The formation of a light aqua colored precipitate was indicative of the formation of the iron-cyanide complex, Figure 8, thus a positive result for cyanide.

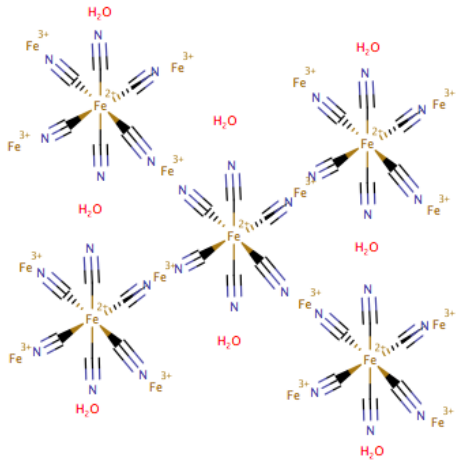


Figure 8: Prussian blue crystal complex. Iron (III) interacts with the corner cyanide groups. Six cyanide groups interact with a central iron (II). Water molecules fill in the gaps of the complex. (Figure was recreated from Buser and coworkers[37]).

The protocol for the Prussian blue test in this study was developed using apple seeds, which are known to contain the cyanogenic glycoside amygdalin. The preliminary Prussian blue test for cyanogenic compounds in wild Maine elderberry seeds produced a light blue/green precipitate, indicative of a positive result, Figure 9. This indicated the presence of cyanogenic glycosides in Maine elderberries.

Figure 9: Light blue/green precipitate indicative of a positive Prussian blue test.

3.2 HPLC-UV and HPLC-ESI/MS Method Development

The positive results from the Prussian blue test qualitatively indicated the presence of cyanogenic compounds in Maine elderberry seeds, so quantification of prunasin and sambunigrin was pursued, beginning with the analysis of prunasin standards, obtained from Sigma-Aldrich. Although unsure of the retention times in the MS total ion chromatogram as well as the UV-chromatogram, the mass-to-charge ratio (*m/z*) for prunasin was expected to be 294 for the anion (ESI^-) and 296 for the cation (ESI^+) when using HPLC-ESI/MS.

3.21 Effect of Solvent Flow Rate on Chromatographic Separation

The speed at which the solvent system is introduced to an HPLC column has an effect on the separation of the injected sample. Some samples separate better at slower flow rates, as the sample has more time to interact with the column, while others do better with faster flow rates because the sample may chemically change over time or eluted

peaks are too broad to analyze accurately. To test the effects of flow rate on the chromatography of our prunasin standard, flow rates of 0.2 mL/min and 0.4 mL/min were examined, Figures 10-13. These flow rates were chosen because when doing HPLC-MS, flow rates under 0.4 mL/min must be used. This is because higher flow rates induce a large pressure on the MS instrument and can cause damage. The 0.4 mL/min flow rate was chosen because it was the fastest that could be used safely, thus taking less time to be analyzed. The 0.2 mL/min flow rate was chosen because it was 0.2 mL/min different than the 0.4 mL/min flow rate, which was a large enough change to allow comparison to be done while still being able to elute prunasin within an hour. Since separation did not improve with the slower flow rate, 0.2 mL/min, a flow rate of 0.4 mL/min was selected for further analysis.

Peaks were confirmed to be due to the solvent system by comparing the UV chromatograms measured at 200 nm and 280 nm. The disappearance of these signals at a higher wavelength, as well as their absence in the HPLC-ESI/MS, indicated that at 200 nm solvents might be observed. These peaks occur before 10 minutes in Figure 10 and before 20 minutes in Figure 12.

The diastereomeric relationship between prunasin and sambunigrin lead us to expect two peaks (two chemically different compounds), which were observed. However, upon further analysis with HPLC-ESI/MS, the less intense peak that appears before each of the prunasin peaks (indicated in each figure) may be due to an impurity, given that the Sigma-Aldrich standard's purity was reported as ≥ 90% and since the m/z=294, prunasin, is not seen in this peak, Figures 11 and 13.

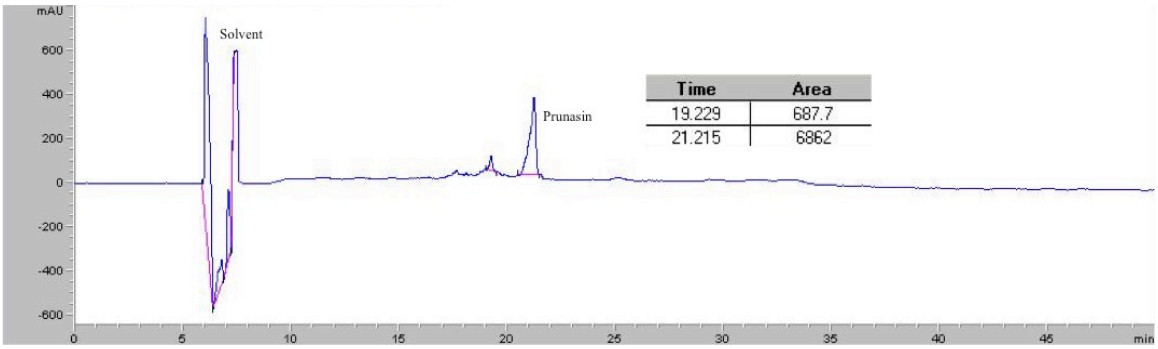


Figure 10: HPLC-UV chromatogram of 0.2 mg/mL prunasin obtained from Sigma-Aldrich. Solvent system A (ultrapure water with 0.05% formic acid and acetonitrile with 0.05% formic acid run at a flow rate of 0.4 mL/min) was used. The peak at 21.215 minutes retention time corresponds to prunasin. Peaks before 10 minutes are due to solvent.

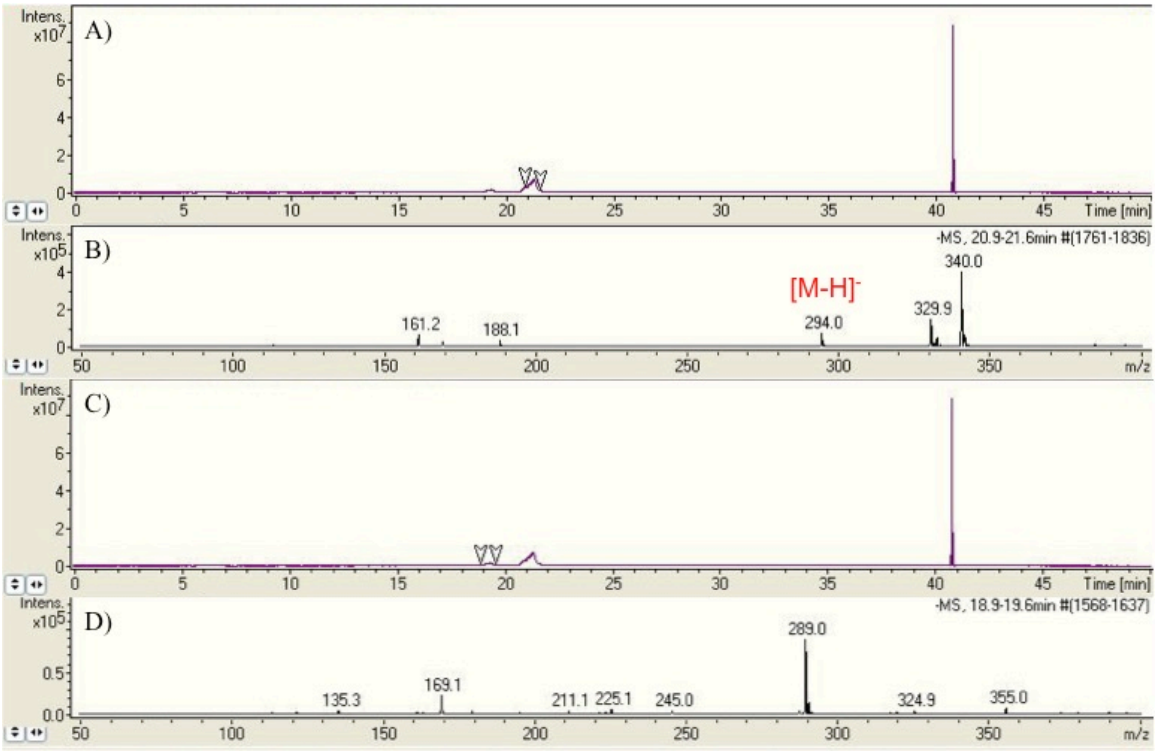


Figure 11: Total ion chromatograms and their MS of 0.2 mg/mL prunasin obtained from Sigma-Aldrich. (A) HPLC-ESI$^-$/MS total ion chromatogram with retention times 20.9-21.6 minutes selected. (B) Average MS of the selected region from A. (C) HPLC-ESI$^-$/MS total ion chromatogram with retention times 18.9-19.6 minutes selected. (D) Average MS of the selected region from C. Solvent system A (ultrapure water with 0.05% formic acid and acetonitrile with 0.05% formic acid run at a flow rate of 0.4 mL/min) was used. The peak at 21.25 minutes retention time corresponds to prunasin, m/z = 294.

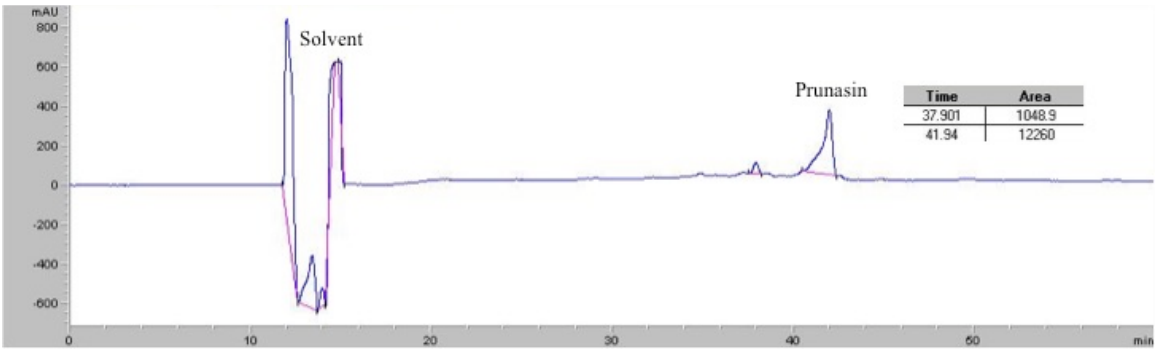


Figure 12: HPLC-UV chromatogram of 0.2 mg/mL prunasin obtained from Sigma-Aldrich. Solvent system B (ultrapure water with 0.05% formic acid and acetonitrile with 0.05% formic acid run at a flow rate of 0.2 mL/min) was used. The peak at 41.94 minutes retention time corresponds to prunasin. Peaks before 20 minutes are due to solvent.

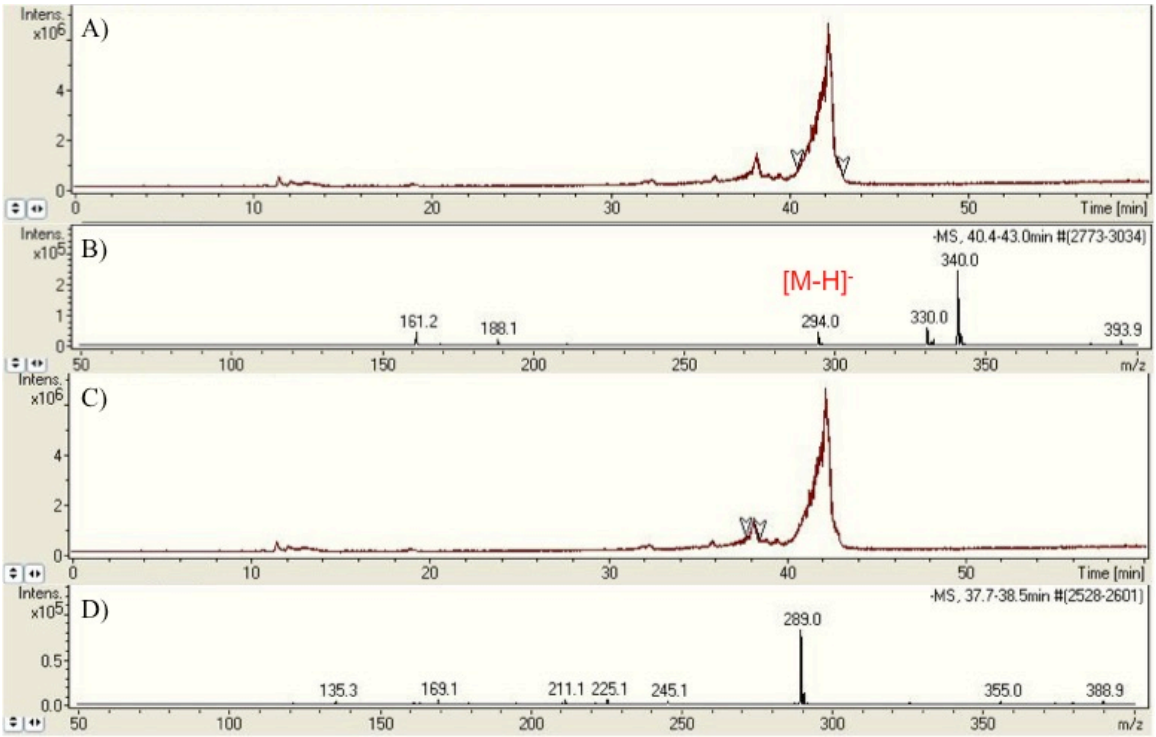


Figure 13: Total ion chromatograms and their MS of 0.2 mg/mL prunasin obtained from Sigma-Aldrich. (A) HPLC-ESI$^-$/MS total ion chromatogram with retention times 20.9-21.6 minutes selected. (B) Average MS of the selected region from A. (C) HPLC-ESI$^-$/MS total ion chromatogram with retention times 18.9-19.6 minutes selected. (D) Average MS of the selected region from C. Solvent system A (ultrapure water with 0.05% formic acid and acetonitrile with 0.05% formic acid run at a flow rate of 0.2 mL/min) was used. The peak at 21.25 minutes retention time corresponds to prunasin, m/z = 294.

3.22 Comparison of HPLC-ESI$^+$/MS to HPLC-ESI$^-$/MS

The presence of unknown *m/z* signals in HPLC-ESI$^-$/MS chromatograms, Figures 11 and 13, lead to the investigation of the identification of these unknown compounds. The first step of ESI/MS is the ionization of material eluted from the HPLC column. Here, the material can either be made into cations (ESI$^+$/MS) or anions (ESI$^-$/MS) by being passed into a high voltage electric field. In previous research on prunasin ESI$^-$ was used, but in this study, we investigated ESI$^+$ to determine which method would be most accurate under this study's particular conditions. No signal was observed in ESI$^+$, and a peak for *m/z* = 294 was not seen, Figure 14. Thus, further ESI/MS analysis was done using ESI$^-$.

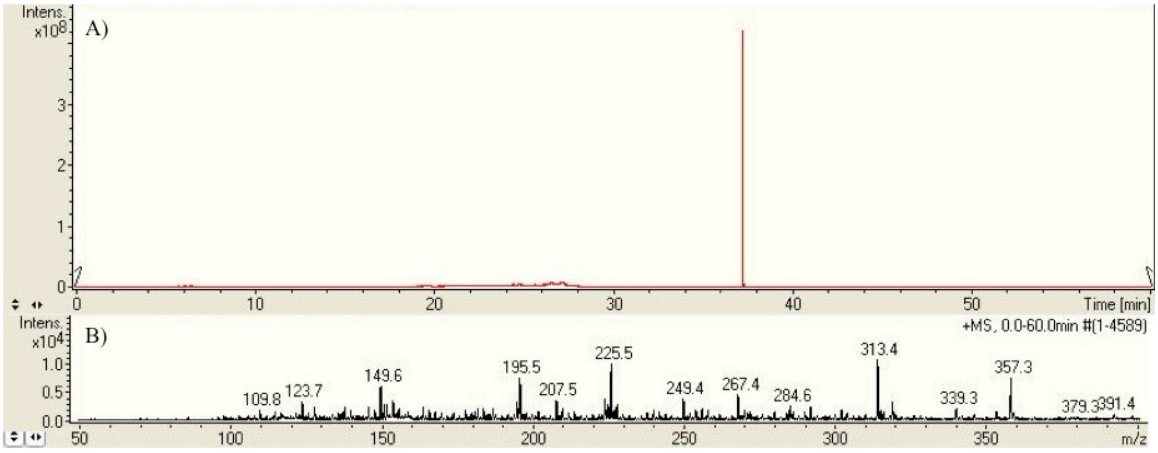


Figure 14: Total ion chromatogram and MS of 0.2 mg/mL prunasin obtained from Sigma-Aldrich. (A) HPLC-ESI$^+$/MS total ion chromatogram. (B) Average MS of A. Solvent system A (ultrapure water with 0.05% formic acid and acetonitrile with 0.05% formic acid run at a flow rate of 0.4 mL/min) was used. The signal at 37 minutes is an artifact.

3.23 Chemical Change of Standard

Prunasin standard from Sigma-Aldrich that had been dissolved in HPLC grade methanol and stored at -20˚C for 66 days was found to have undergone a chemical

change where prunasin could not be detected using the same parameters used previously. Instead, no distinct peaks could be identified in the HPLC-UV or HPLC-ESI$^-$/MS chromatograms, Figures 15-16. This change may have been degradation of prunasin or the reaction of prunasin with impurities in the standard. Although the lack of distinct signals in Figures 15 and 16 indicate degradation, new products formed from the reaction of prunasin and impurities may not absorb at 200 nm, and may also be incompatible with ESI/MS. Thus, determining the change is not possible with these results alone. These results led to the purchase of new prunasin standard from a different supplier, Santa Cruz Biotechnology (Santa Cruz, CA, USA). These changes could be from the degradation of prunasin or from the reaction of prunasin with impurities in the standard.

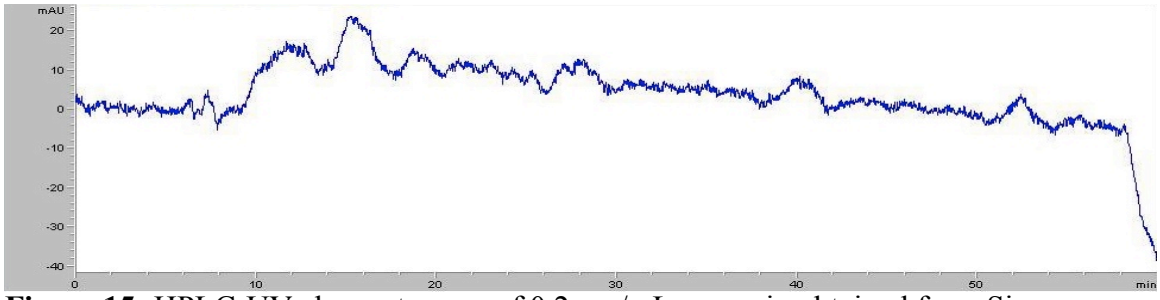


Figure 15: HPLC-UV chromatogram of 0.2 mg/mL prunasin obtained from Sigma-Aldrich that had been in solution for 66 days. Solvent system A (ultrapure water with 0.05% formic acid and acetonitrile with 0.05% formic acid run at a flow rate of 0.4 mL/min) was used.

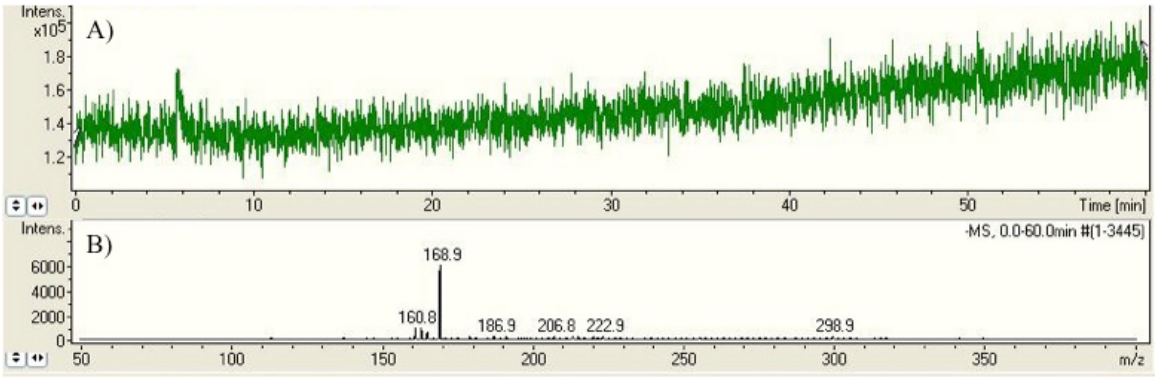


Figure 16: Total ion chromatogram and MS of 0.2 mg/mL prunasin obtained from Sigma-Aldrich. (A) HPLC-ESI⁻/MS total ion chromatogram. (B) Average MS of A. Solvent system A (ultrapure water with 0.05% formic acid and acetonitrile with 0.05% formic acid run at a flow rate of 0.4 mL/min) was used.

3.24 MS/MS of an Impurity in the Standard

Although prunasin, *m/z*=294, was seen in the HPLC-ESI⁻/MS, other compounds with *m/z*=340 and *m/z*=330 were more intense, Figures 11B and 13B. This means that these compounds were in greater quantity than prunasin. With the correct *m/z* value, the compound with *m/z*= 340 was hypothesized to be the addition of formic acid to prunasin. Thus, a fragmentation of this ion (MS/MS) was conducted, Figure 17. This process dissociates the parent compound (*m/z*=340) into smaller fragments so that the identity of the parent can be determined. When MS/MS of *m/z*=340 was done, prunasin (*m/z* =294) is seen suggesting that the prunasin had undergone a chemical change at some point to form this parent ion (*m/z*=340). Since the *m/z*=340 is larger than the *m/z* value corresponding to prunasin, this parent ion is not due to the degradation of prunasin. This compound could be the product of either formic acid or an impurity in the standard bonding with the prunasin. Another possibility is that this compound is an impurity in the

sample. We were not able to identify the other fragments, *m/z*=187.9 and 160.9, nor the parent ion.

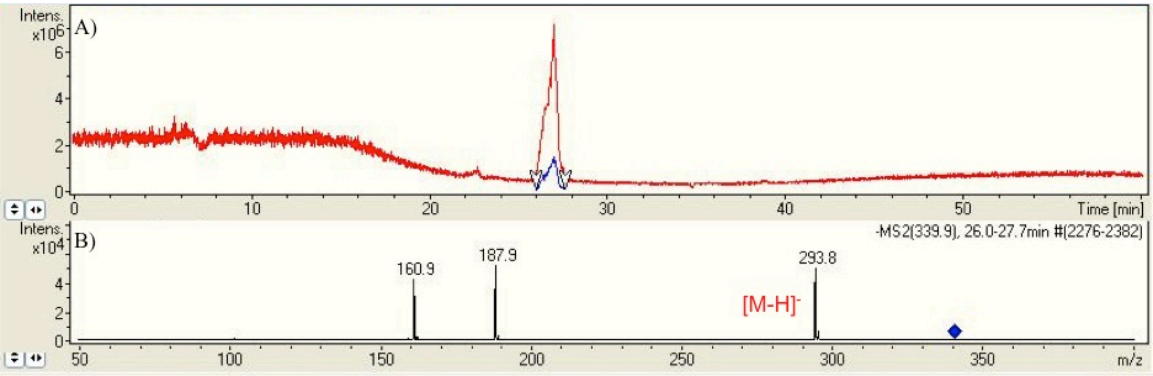


Figure 17: Total ion chromatogram and MS of 0.2 mg/mL prunasin obtained from Santa Cruz Biotechnology. (A) HPLC-ESI⁻/MS total ion chromatogram. (B) MS/MS of *m/z*=340 from A. Solvent system A (ultrapure water with 0.05% formic acid and acetonitrile with 0.05% formic acid run at a flow rate of 0.4 mL/min) was used.

3.25 Effect of Acid in Solvent System

The possibility of prunasin binding to formic acid to form the compound with *m/z*=340 initiated the analysis of using different acids in the mobile phase to see if this compound would be present when a different acid was used. When using HPLC-ESI/MS, an acid must be present in the solvent system to help ionize the sample. Acids also can make HPLC-UV peaks sharper in some cases, allowing for more accurate analysis. However, acids can react chemically with samples by catalyzing reactions or protonating compounds. This reaction with acid could lead to inaccurate results while using HPLC-ESI/MS or HPLC-UV as compounds not in the sample would be detected. We believed that the compound with *m/z*=340 could be due to formic acid binding to prunasin. Thus, we tested solvent systems with different acids by replacing the 0.05% formic acid in the mobile phase with trifluoroacetic acid (0.01%, 0.1%, and 0.05%), Figures 18-19. This

was done to examine both the compound with *m/z*=340 as well as the effects of acid on the separation of prunasin, and if the acid is binding to prunasin.

In Figures 19A and 19F, noise is seen. If the ESI/MS detector is unable to detect different compounds (many compounds carry similar *m/z* values) noise may be experienced where there are many sharp signals throughout the total ion chromatogram. At 0.01% trifluoroacetic acid, the ESI^-/MS signal was noisy (Figure 19A), indicating under-ionization. The acid concentration was increased to 0.1%, which resulted in less noise (Figure 19C); however, no prunasin could be detected indicating that the high acid concentration may have caused the degradation of prunasin. Further, the absence of noise may have been due to the artifact signal at 29.6 minutes, which caused the scale of the intensity axis to be larger in 19C than in 19A and 19E. This would make all other signals appear smaller, and reduce the appearance of noise. The concentration of trifluoroacetic acid was reduced to 0.05%, which was again noisy, Figure 19E. These results indicated that the trifluoroacetic acid may be too strong of an acid to use and that it degrades prunasin.

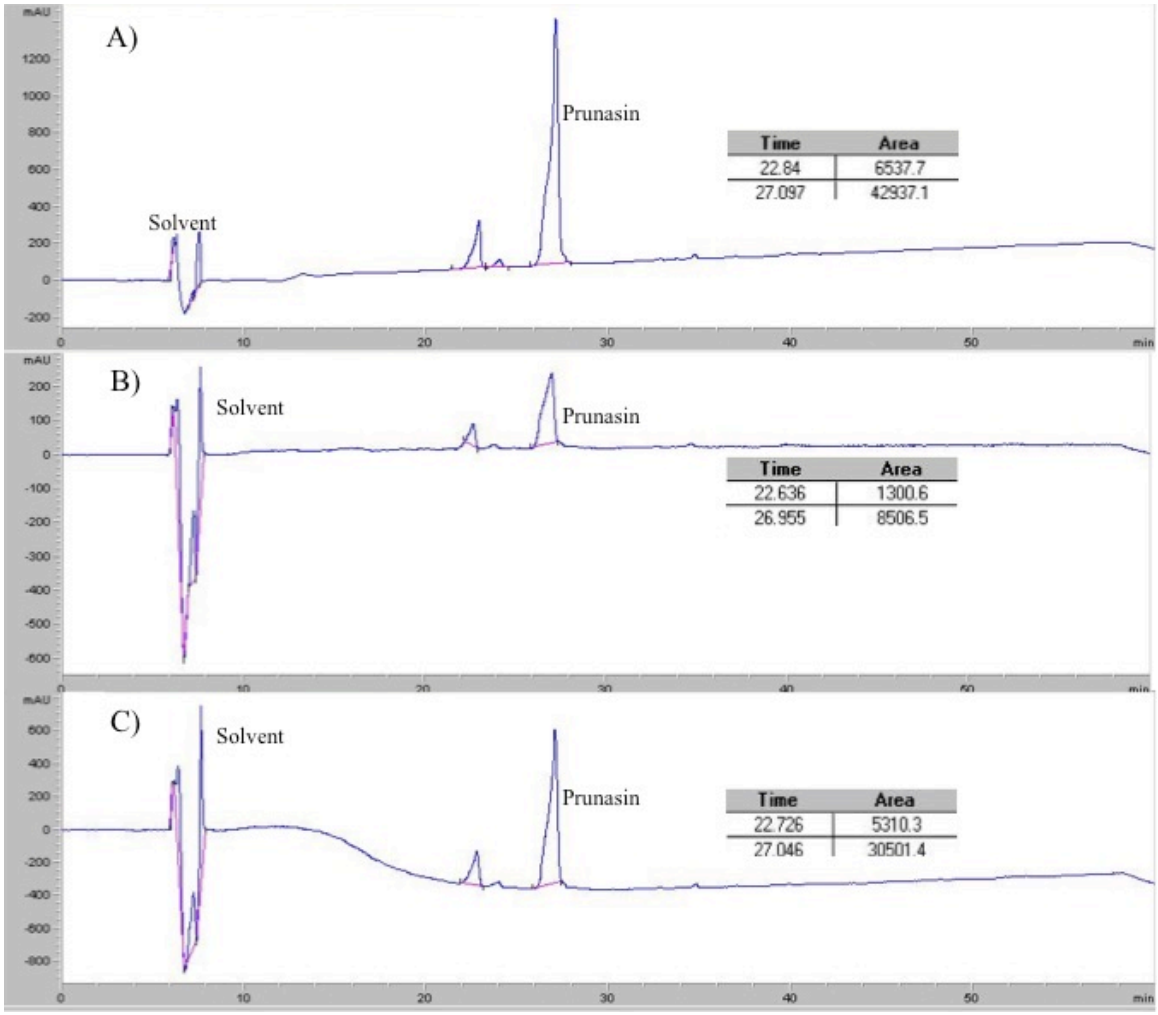


Figure 18: HPLC-UV chromatograms of 0.5 mg/mL prunasin obtained from Santa Cruz Biotechnology in three solvent systems: (A) System C (ultrapure water with 0.01% trifluoroacetic acid and acetonitrile with 0.01% trifluoroacetic acid), (B) System D (ultrapure water with 0.1% trifluoroacetic acid and acetonitrile with 0.1% trifluoroacetic acid), and (C) System E (ultrapure water with 0.05% trifluoroacetic acid and acetonitrile with 0.05% trifluoroacetic acid).

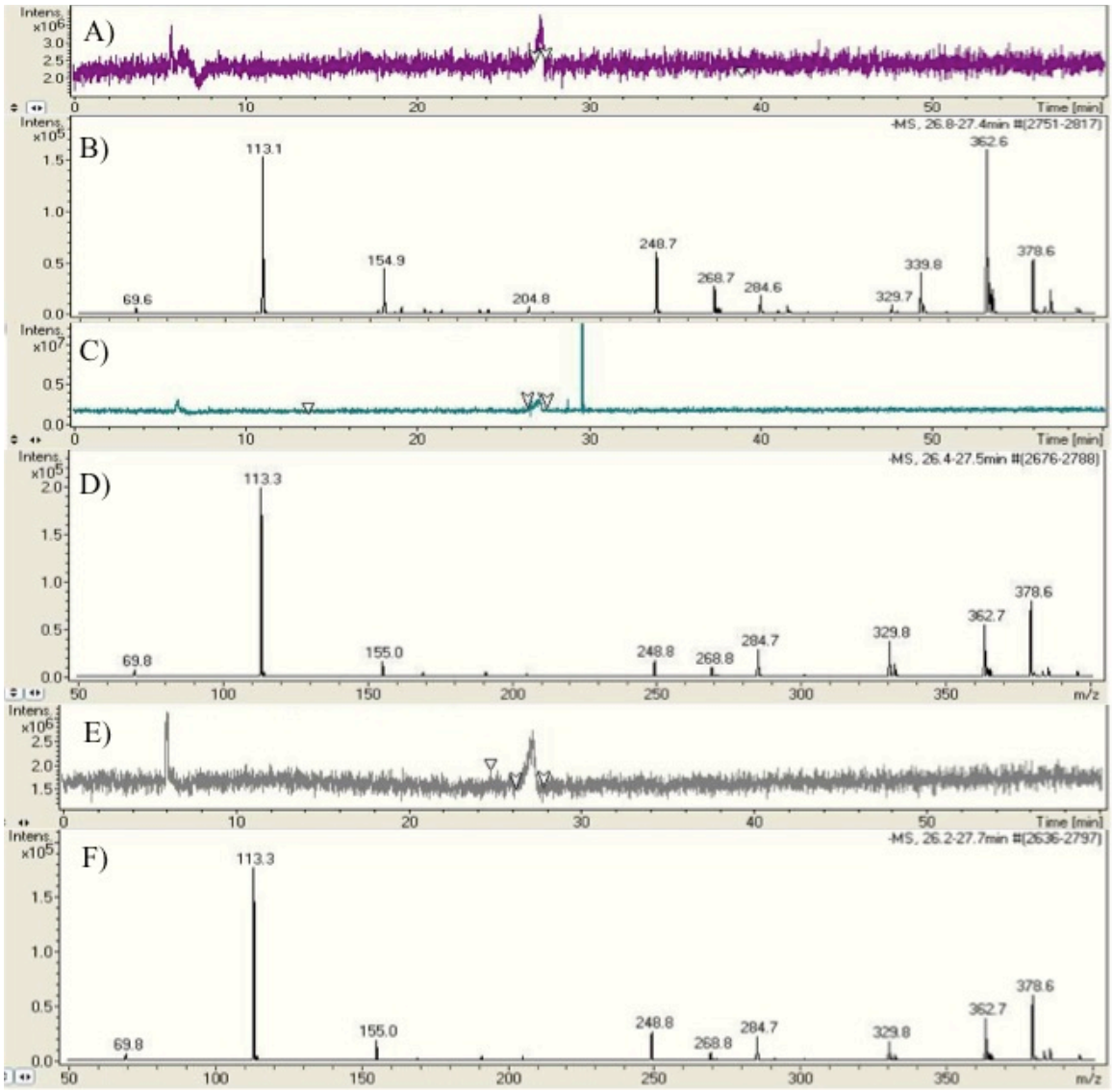


Figure 19: HPLC-ESI/MS total ion chromatograms and their MS of 0.5 mg/mL prunasin obtained from Santa Cruz Biotechnology in three solvent systems. (A) Total ion chromatogram of system C (ultrapure water with 0.01% trifluoroacetic acid and acetonitrile with 0.01% trifluoroacetic acid). (B) Average MS of the selected region in (A). (C) Total ion chromatogram of system D (ultrapure water with 0.1% trifluoroacetic acid and acetonitrile with 0.1% trifluoroacetic acid). (D) Average MS of the selected region in (C). (E) Total ion chromatogram of system E (ultrapure water with 0.05% trifluoroacetic acid and acetonitrile with 0.05% trifluoroacetic acid). (F) Average MS of the selected region in (E).

Since trifluoroacetic acid was found to be too strong of an acid (pKa 0.3) to use in this study acetic acid, a weaker acid (pKa 4.76), was tested, Figures 20-21. As the pKa of formic acid is 3.75, acetic acid is also weaker than formic acid. Although the *m/z* value of prunasin was seen while using acetic acid, Figure 21B, the compounds with *m/z*=330 and 340 were still more predominant than prunasin, meaning that the unknown compounds were in a larger quantity than prunasin. A consistent baseline was also difficult to achieve, shown in the downward slope occurring in the HPLC-UV chromatogram at 29 minutes, Figure 20. This shift in the baseline could be due to a change in column pressure, which could be caused by a buildup of materials (compounds remaining in the column over time) in the column.

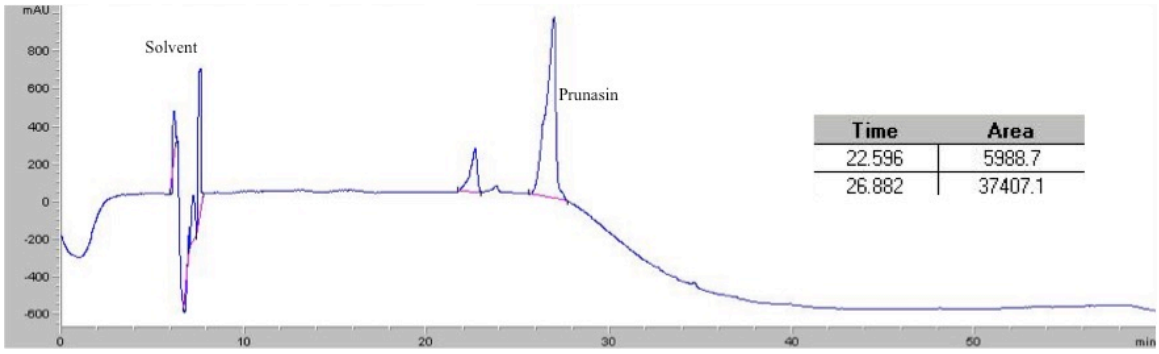


Figure 20: HPLC-UV chromatogram of 0.5 mg/mL prunasin obtained from Santa Cruz Biotechnology. Solvent system F (ultrapure water with 0.02% acetic acid and acetonitrile with 0.02% acetic acid run at a flow rate of 0.4 mL/min) was used. The peak at 26.882 minutes retention time corresponds to prunasin.

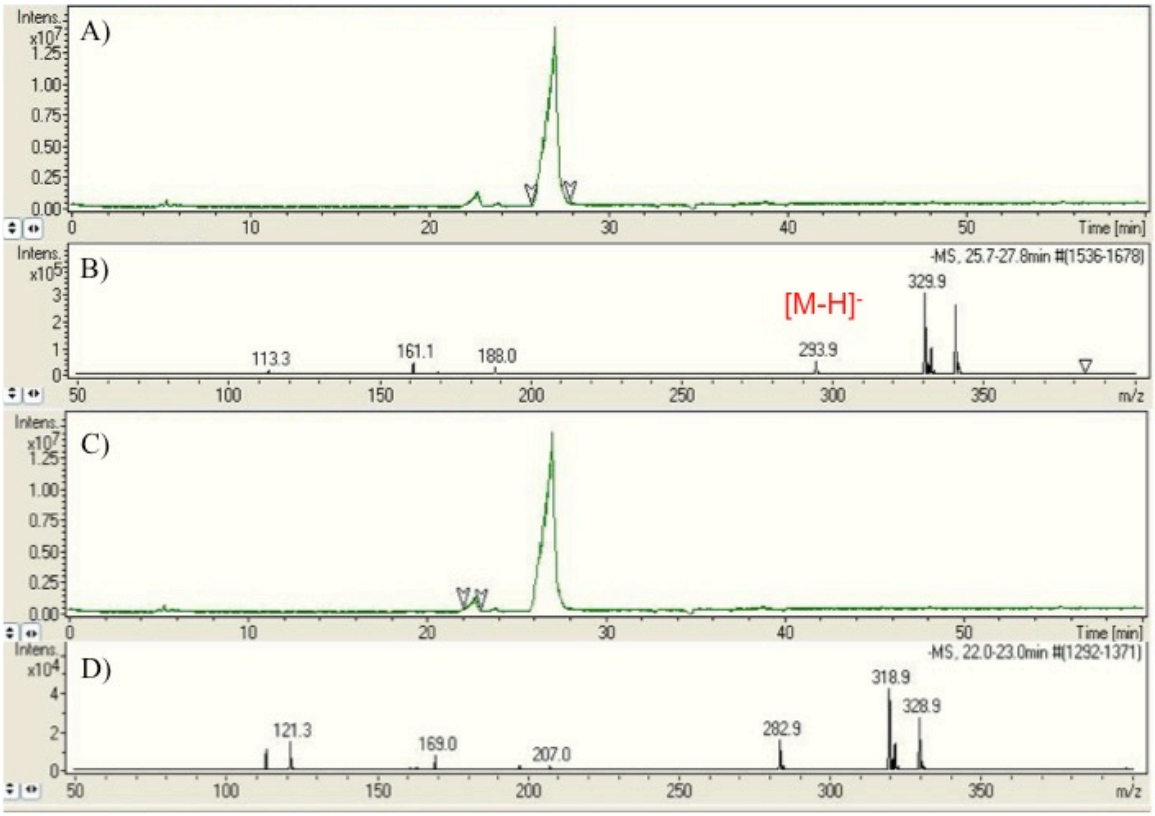


Figure 21: HPLC-ESI/MS total ion chromatograms and their MS of 0.5 mg/mL prunasin obtained from Santa Cruz Biotechnology. (A) HPLC-ESI$^-$/MS total ion chromatogram with retention times 25.7-27.8 minutes selected. (B) Average MS of the selected region from A. (C) HPLC-ESI$^-$/MS total ion chromatogram with retention times 22.0-23.0 minutes selected. (D) Average MS of the selected region from C. Solvent system F (ultrapure water with 0.02% acetic acid and acetonitrile with 0.02% acetic acid run at a flow rate of 0.4 mL/min) was used. The peak at 26.75 minutes retention corresponds to prunasin, m/z = 293.9.

3.26 HPLC-UV with an Acid-Free Solvent System

At this point, it was decided to conduct further analysis with UV detection only. This decision allowed the omission of acid from the solvent system, Figure 22. Acid was able to be omitted from the solvent system when HPLC-ESI/MS was not conducted because ionization is not done when using HPLC-UV. Due to the catalytic effect of acids, the purpose of omitting acid from the solvent system was to prevent, or slow, the chemical changes in the prunasin standard.

The absence of acid gave sharper peaks with no tails, Figure 22. Because flow rates exceeding 0.4 mL/min induce a pressure that is large enough to cause damage to the ESI/MS detector and/or the HPLC system, all flow rates used with our HPLC-ESI/MS were ≤ 0.4 mL/min. Thus, the faster flow rate of 1.0 mL/min could only be used after the ESI/MS analysis was completed, that is only HPLC-UV detection used.

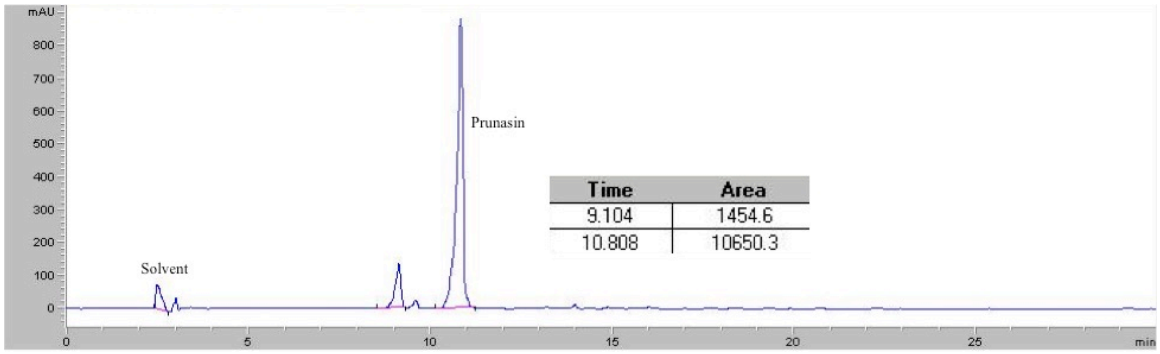


Figure 22: HPLC-UV chromatogram of 0.3 mg/mL prunasin obtained from Santa Cruz Biotechnology. Solvent system G (ultrapure water and acetonitrile run at a flow rate of 1.0 mL/min) was used. The peak at 10.808 minutes retention time corresponds to prunasin.

3.27 Characterization of Change in Standard Over Time when Dissolved in Methanol and as a Solid

In the course of this study, it was found that the 0.3 mg/mL prunasin standard had chemically changed after being stored in HPLC grade methanol for three weeks at -20˚C. This change initiated the characterization of how the standard changes over time. A new 0.5 mg/mL prunasin standard was prepared in methanol and analyzed by HPLC-UV on day 0 and day 2 after being left at room temperature. Due to power loss, analysis on day 1 could not be conducted. The standard was removed from methanol after analysis on day 2 by streaming nitrogen gas over the solution to evaporate the methanol. The solid prunasin was left at room temperature for three days. On day three, the solid prunasin was

re-dissolved in HPLC methanol and analyzed using HPLC-UV. These chromatograms, Figure 23, show that the peak area of prunasin decreased more over the 48-hour span while in methanol than it did three days after being removed from solution. This suggests that more chemical change of the prunasin standard (degradation or reaction with impurities) occurs when the prunasin is dissolved in methanol than when left as a solid. Comparing the area of the peaks at 10.8 minutes retention time in Figure 23A-C allowed for the calculation of decrease in prunasin, as a decrease in these peaks was assumed to be due to a loss of prunasin, Table 2.

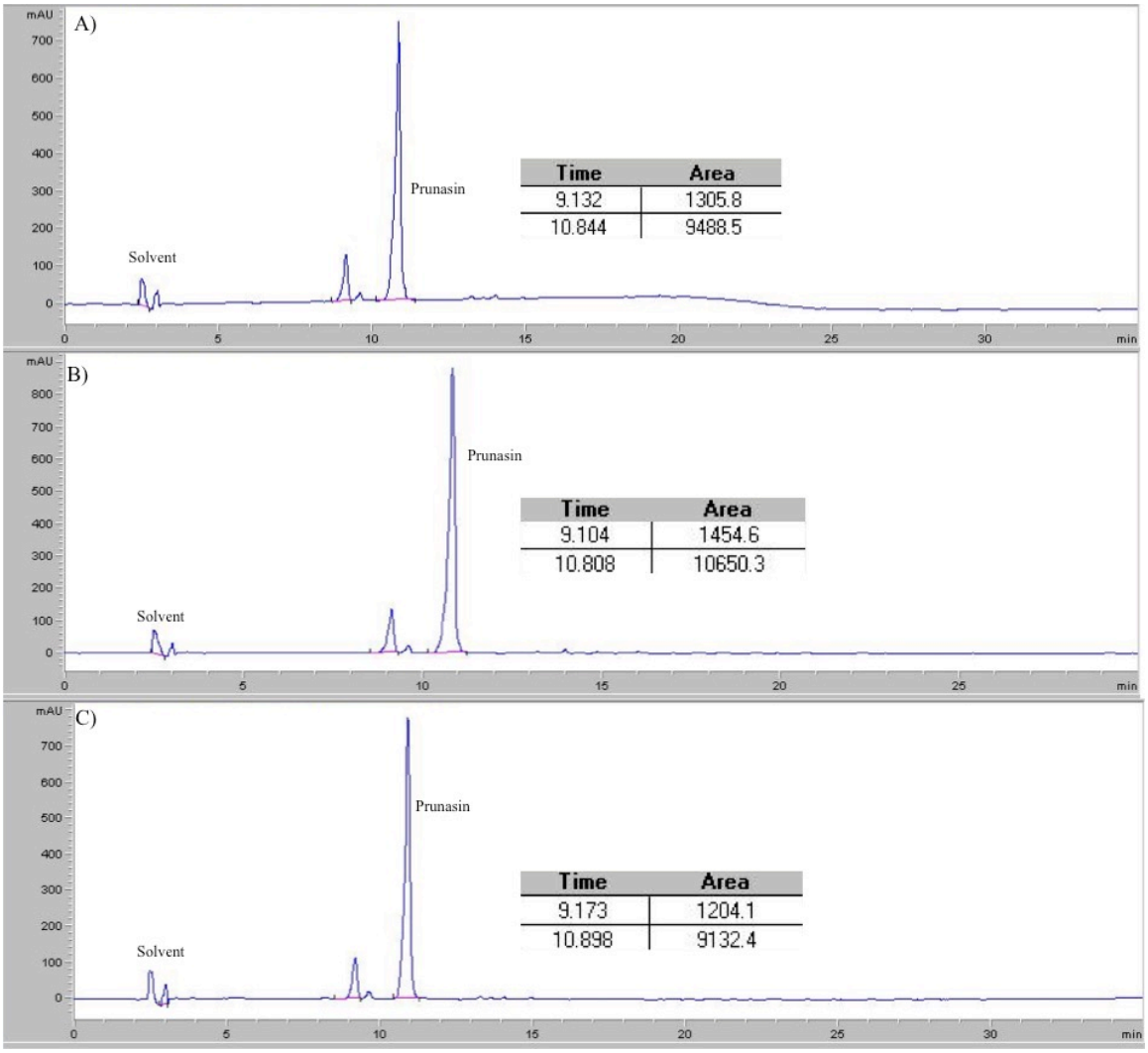


Figure 23: HPLC-UV chromatogram of 0.3 mg/mL prunasin obtained from Santa Cruz Biotechnology (A) on day 0 of suspension in methanol, (B) two days post suspension in methanol, and (C) re-dissolved after three days. Solvent system G (ultrapure water and acetonitrile run at a flow rate of 1 mL/min) was used. Peaks at 10.8 minutes retention time correspond to prunasin.

Table 2: Changes in prunasin over time. The percent decreased represents the change in peak area compared to the peak area of the previous sample.

Sample	Area of Peak	Decrease (%)
Day 0	10650.3	-
Day 2	9488.5	10.909
Re-Dissolved	9132.4	3.7530

Contact with a Sigma-Aldrich technology support person informed our group that UV-Vis detectors should not be used on their prunasin standard because an impurity in their compound absorbs more intensely than prunasin at 200 nm, which is the wavelength of maximum absorption by prunasin. They suggest that instead, an Evaporative Light Scattering Detector (ELSD) must be used. A recently obtained Ultra Performance Liquid Chromatogram (UPLC) on our campus, with an ELSD, will be useful for this analysis in a future study.

3.3 HPLC-UV and HPLC-ESI⁻/MS of Elderberries

While waiting for the arrival of prunasin standard from Santa Cruz Biotechnology, work on elderberries was initiated to be time efficient. Extractions were made from the seeds, new and old canes, and leaves of the elder plants. Although HPLC-UV was attempted on the new stems as well as the leaves, these chromatograms could not be analyzed for prunasin because a flat baseline was not achieved. This indicated that there might be compounds in these samples retained in the HPLC column, which would explain the inability to obtain a flat baseline. These baselines made analysis difficult, as signals in the chromatograms that were due to compounds were unable to be distinguished. For this reason, these chromatograms are omitted from this report. Focus was directed towards seed extracts, due to time constraints, which gave usable spectra. Three extraction methods, refluxing, sonication, and stirring (described in section 2.7) were analyzed, Figures 24A-C. These methods were chosen because they offer three different temperature conditions for getting prunasin into solution. Refluxing uses the highest temperature, 60˚C, sonication uses a more moderate temperature, 30˚C, and

stirring uses the lowest temperature, 20˚C. As there was no peak at the retention time expected for prunasin (21.2 minutes), in Figures 24A-C, it is unlikely that prunasin was present.

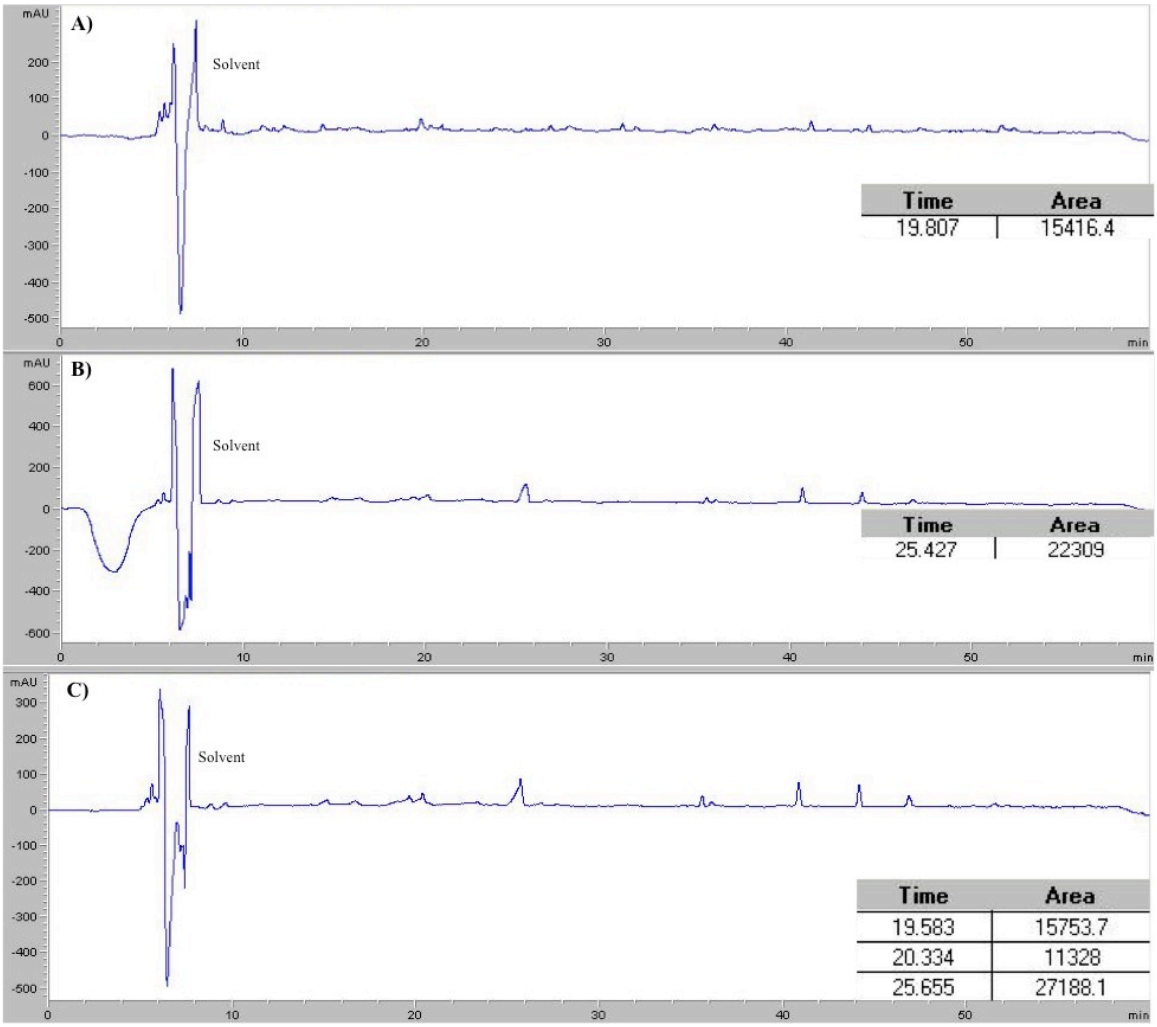
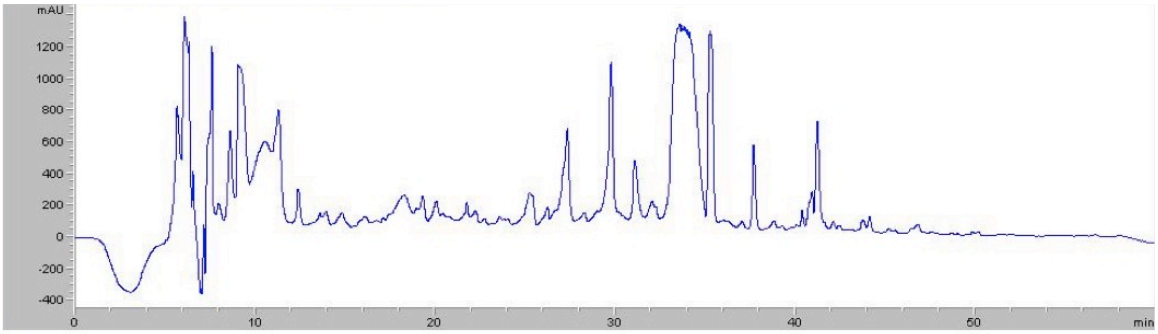


Figure 24: HPLC-UV chromatograms of cyanogenic glycosides extracted from Maine elderberry seeds using (A) refluxing, (B) sonication, and (C) stirring. Description of extractions can be found in the methods section. Solvent system A (ultrapure water with 0.05% formic acid and acetonitrile with 0.05% formic acid run at a flow rate of 0.4 mL/min) was used.

The lack of a peak at 21.2 minutes retention time in Figures 24A-C, where prunasin was expected based on results from the standard, suggested that manual extraction of seeds before analysis may induce prunasin hydrolysis. In an attempt to

eliminate the manual separation of seeds from the analysis procedure, extractions on whole elderberries were completed and analyzed. Although the UV chromatogram did not show a major peak at 21.2 minutes retention time, Figure 25, HPLC-ESI⁻/MS showed the presence of a compound with *m/z* = 293.8 (Figure 26F), suggesting the presence of prunasin. The retention time of this peak (containing the compound with *m/z*=293.8) had a retention time of 18.5 minutes. This retention time was earlier than that shown by prunasin standards, which experience a retention time of 21.2 minutes for prunasin. However, inconsistencies in results due to chemical change experienced with the standard prevented us from eliminating this *m/z*=294 in Figure 26F from being identified as prunasin because the reactivity of prunasin in solution was not understood.



Time	Area
8.57	24853.5
9.081	39493.3
10.245	28754.4
10.51	18655.9
11.25	45726.4
12.365	22953.5
18.208	37781
19.257	13175.2
27.3	33818.8

Time	Area
29.76	41600.9
31.079	25326.8
33.387	40353.3
34.059	13037.7
34.18	41622.3
35.223	15318.6
35.289	15997.5
37.665	16199.1
41.2	19750.4

Figure 25: HPLC-UV chromatogram of whole elderberries extracted by reflux. Solvent system A (ultrapure water with 0.05% formic acid and acetonitrile with 0.05% formic acid run at a flow rate of 0.4 mL/min) was used.

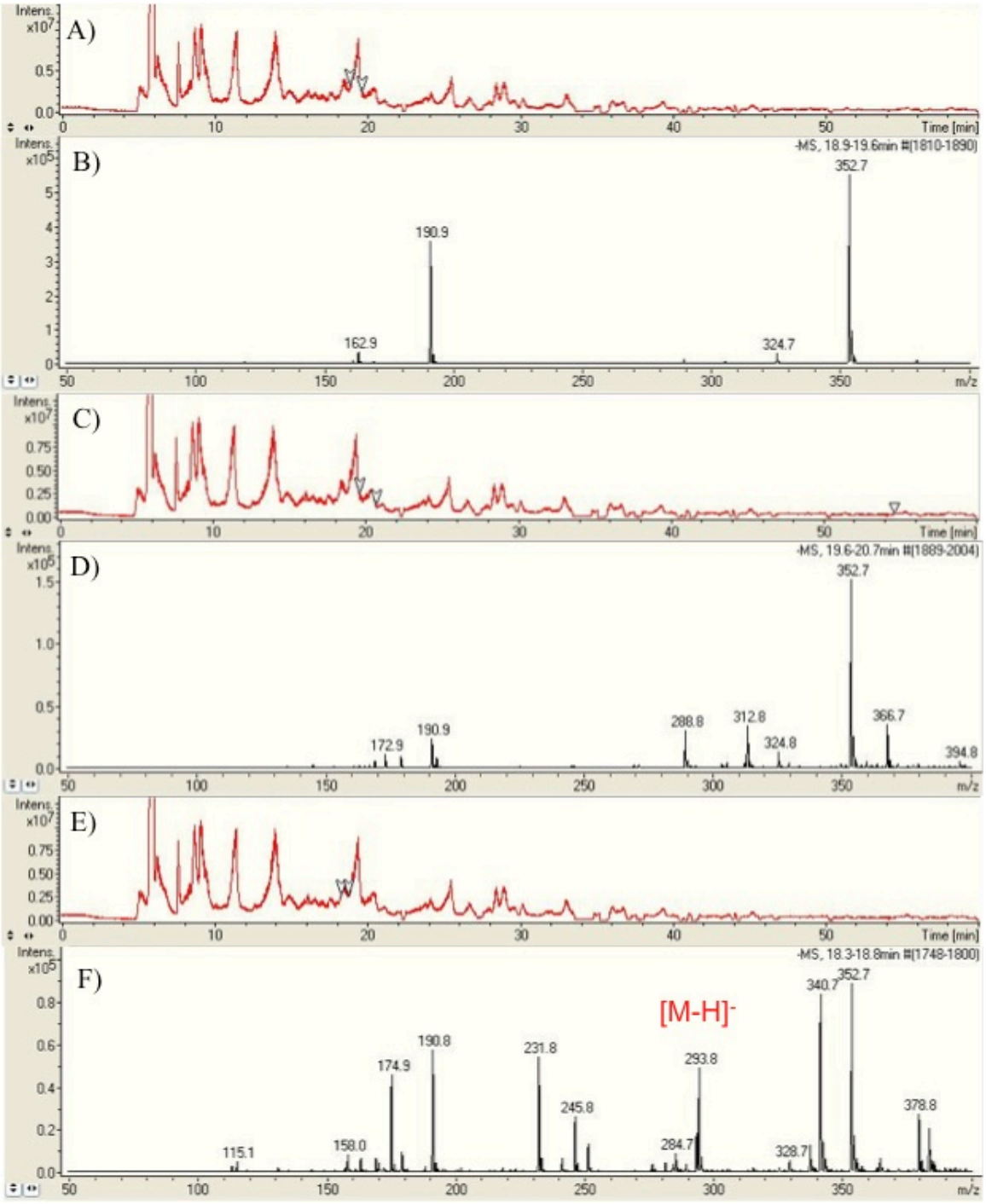


Figure 26: HPLC-ESI$^-$/MS of whole elderberries extracted by reflux. The average mass spectra of selected regions (B, D, F) are provided below the total ion chromatograms (A, C, E). Solvent system A (ultrapure water with 0.05% formic acid and acetonitrile with 0.05% formic acid run at a flow rate of 0.4 mL/min) was used.

To analyze the presence of a compound with the same *m/z* value as prunasin in the whole berry, further work on this extract was done. These whole berries, containing cyanogenic glycosides extracted 27 days previously by the reflux method, were spiked with 0.3 mg/mL of Santa Cruz Biotechnology prunasin standard in a 5:2 ratio (extract: standard), Figure 27. The peak at 10.863 minutes retention time in Figure 27-B is absent in Figure 27-A, indicating that prunasin was not in the 27-day old whole berry extract as this peak only appeared when prunasin was added to the sample. This sample had been stored under the same conditions as the prunasin standard, at -20˚C in HPLC grade methanol. The absence of prunasin may be because the extract was so old that the prunasin underwent the same chemical change as the standard.

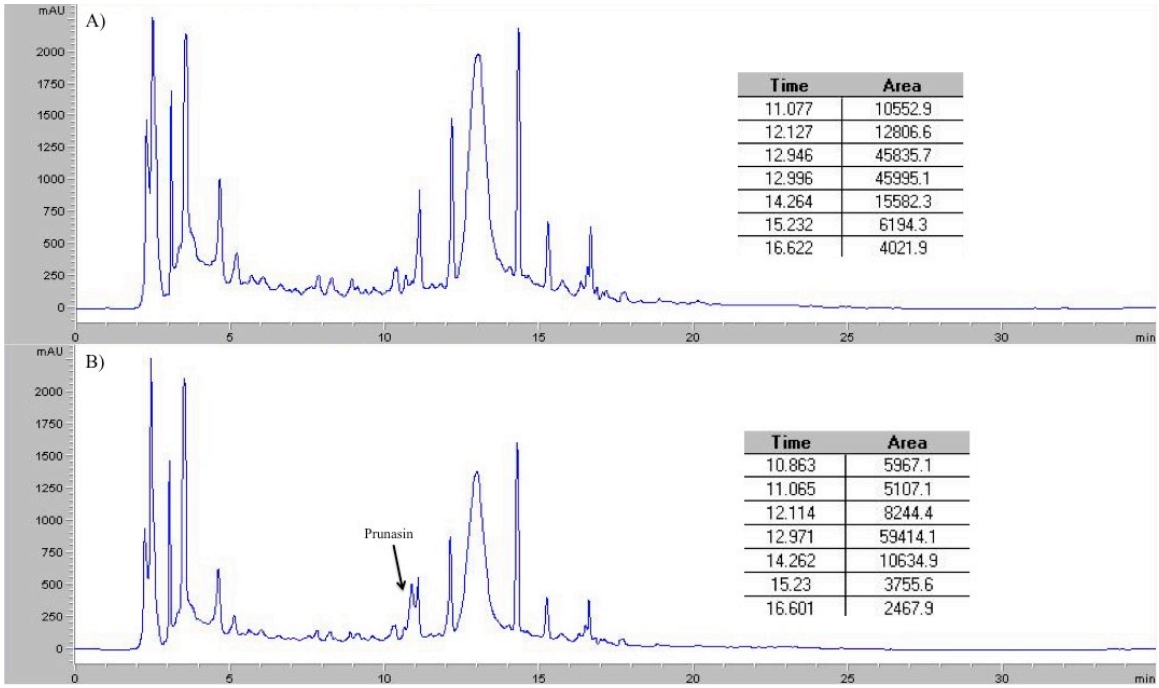


Figure 27: HPLC-UV chromatogram of prunasin spiked whole elderberry cyanogenic glycoside extract 27 days after reflux extraction. A) extract before spiking, B) extract post spike. Solvent system G (ultrapure water and acetonitrile run at a flow rate of 1 mL/min) was used. The peak at 10.863 minutes retention time in (B) corresponds to prunasin.

4. Conclusions and Future Work

The lack of research on the toxicology of the elderberry motivated the quantification of the cyanogenic glycosides in Maine elderberries. Based on the results of this study, we propose the following conclusions; first, our prunasin standard underwent chemical change over time when dissolved in HPLC grade methanol and stored at -20˚C. This study saw that when stored as a solution in methanol, the UV-chromatogram peak areas of the standard decrease over two days and over two months the signals from HPLC-UV and ESI/MS analysis disappear. This reduction means that the standard is chemically changing. One possible change is that prunasin is degrading. Another possibility is that the impurities in the standard are reacting with prunasin, forming new products. More work is needed to investigate what this change is because although the data collected in this study suggest degradation, due to the absence of distinct peaks in the UV and MS chromatograms, new products formed from the reaction of prunasin with impurities may not be able to be detected under these conditions. New products may be unable to absorb 200 nm wavelength light, and may have *m/z* values outside of the range selected for this study (50-400 *m/z*). This change exemplifies the sensitive nature of biological molecules and highlights the need to take caution when storing them.

Second, removal of acid from the mobile phase produced sharper peaks in HPLC-UV chromatograms. This indicates that when using a C_{18} column acid hinders the separation of prunasin standard, causing broader peaks. Thus, acid-free mobile phases should be used for analysis.

Finally, the presence of unknown peaks in HPLC-UV and ESI/MS spectra, as well as notification from Sigma-Aldrich, show that an ELSD must be used as opposed to a UV detector because the impurities in the standard absorb more strongly at 200 nm, which is the wavelength of maximum absorption for prunasin.

Future studies with prunasin should be conducted using an ELSD as recommended by Sigma-Aldrich to ensure maximum detection of prunasin as opposed to that of impurities. Stock solutions for calibration curves should be made immediately before analysis, so as to prevent chemical changes in the standards. Further, different solvents should be analyzed to determine if another, possibly a polar aprotic solvent in which prunasin would still be soluble in but may behave differently in, would not experience the chemical changes that occurred when using methanol. Finally, cyanogenic glycosides from elder samples should be extracted and analyzed on the same day, before hydrolysis of cyanogenic glycosides can occur, so as to quantify the prunasin and sambunigrin accurately.

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