Don’t Be Blue, There’s A New Elderberry on the Scene:
Exploring the Fruit and Flower of the Blue Elderberry (*Sambucus nigra* ssp. *cerulea)*

By

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Abstract

The blue elderberry (*Sambucus nigra* ssp. *cerulea*) is a fruit-bearing shrub native to the western region of North America. Compared to the well-studied European black elderberry (*S. nigra* ssp. *nigra*) and the American elderberry (*S. nigra* ssp. *canadensis*), this subspecies has not been thoroughly analyzed and thus is not used in commercial products. The composition of the fruit and flower, particularly the phenolic profile, was studied for the first time. The blue elderberry has a significantly lower level of anthocyanins than the other subspecies but contains cyanidin 3-sambubioside and cyanidin 3-glucoside like European elderberry. The elderflower of the blue elder is unique in terms of phenolic and volatile profile. Isorhamnetin 3-rutinoside was the dominant phenolic compound in the elderflower, unlike rutin in European and American elderflower. Furthermore, methyl eugenol was one of the most concentrated volatile compounds in the elderflower, a compound that has not been identified in other studies on the European elderflower. These studies have provided more information on the native plant, which has several environmental benefits such as improving air and soil quality when planted in hedgerows, which may facilitate more use of the fruit and flower in commercial products.

Chapter 1 presents information on the composition of the blue elderberry, including the sugar, and acid content of the fruit, and the phenolic profile of the blue elderberry.

Chapter 2 explores the elderflower of the blue elderberry, investigating the volatile and phenolic profile of the flower.

Chapter 3 examines the cyanogenic compounds as well as the impact of thermal processing on the important phenolic compounds present in blue elderberry juice.

INTRODUCTION

Elderberry (*Sambucus nigra* L.) is a part of the Viburnaceae family and grows all over the world, including Europe, North America, and Asia.[1,2] Due to the vast geographic and morphological variety within *Sambucus,* there have historically been many species within the genus. However, a reorganization by Bolli (1994) reclassified some of the most common species under *Sambucus* into subspecies of *S. nigra.*[3] More recently, elderberry was moved out of the Adoxoaceae family, which had already been changed before when elderberry was taken out of the Caprifoliaceae family. These changes have impacted the three subspecies most of interest in this work: the European elderberry *S. nigra* ssp. *nigra*; the American elderberry *S. nigra* ssp. *canadensis*; and the blue elderberry *S. nigra* ssp. *cerulea* (which has recently been argued to not be a subspecies of *S. nigra* due to morphological differences).[4,5] However, due to wide acceptance of this naming scheme for the subspecies, it will be used through this work to align with the current naming, but previous works cited may use the former species names. Furthermore, some sources refer to the entire plant as an "elder", while others refer to the plant as "elderberry", which is also used to denote the fruit of the plant. In this work, "elderberry" is used to discuss the plant as well as the fruit. "Elderflower" is used to refer to the blossoms of the plant.

European elderberry (*S. nigra* ssp. *nigra*) is the most well-studied and widely used subspecies of elderberry in the market. This subspecies grows throughout the European continent, including countries such as Slovenia, Portugal, and Austria. [6–8] The fruit and flower have been studied for decades for their composition and bioactivity, and while elderberry and elderflower are not new ingredients to the market, they have garnered more attention in the last several years as consumers look for more natural remedies and supplements to support their health. This has been especially true during the COVID-19 pandemic, in which elderberry became a popular ingredient

in immunity-supporting supplements.[9] Thus, investigating other elderberry subspecies like the blue elderberry, the focus of future chapters, allows for farmers in the United States to capitalize on this demand, but more information is needed on this particular plant if it is going to be used in consumer products.

FOLKLORE AND HISTORIC USES

There is a long, rich history of the use of different parts of the elderberry plant by many cultures. For example, the wood has been used for kindling and musical instruments. Indeed, the name of the plant is derived from various ancient words related to instruments. The flowers and berries have been used in a variety of beverages, foods, and other herbal supplements.[10] Folklore has many stories about the healing power of the elderberry and elderflower. The plant itself has been revered by many cultures, with a story about the "Elder Mother" living within the plant would protect those near the plant. It was even expected to ask the Elder Mother for the berries or flowers before taking them; without permission, she may seek revenge. The leaves, branches, flowers, and berries were believed to have protective powers for a home and the leaves were also used during burial rituals by some Celtic people. The personification and deep reverence for the elderberry show the importance of the plant through generations. Hippocrates and Pliny the Elder both wrote about elderberry and its medicinal properties.[10] Tribes indigenous to North America used flowers and fruit for medicinal and beverages.[11–13] Berries were also used as a natural dye for baskets and branches were used to make musical instruments.

Elderberry Cultivation

Elderberry is a perennial, deciduous plant native to many regions of the northern hemisphere.[14] Elderberry plants are neither tree nor bush, as the plant sends new canes up each season, which without pruning, can lead to a large, shrub-like plant that can be several meters tall and wide.[15] They prefer to grow in sunny, riparian climates with moist, well-drained soil, though subspecies in North America can be drought-tolerant.[15,16] While pruning even down to the ground level of the elderberry can improve yield and accessibility for harvesting[12], there is a limitation on pruning of the blue elderberry (*S. nigra* ssp. *cerulea*) in the Central Valley of California. Due to the threatened status of the Valley Elderberry Longhorn Beetle (*Desmocerus californicus dimorphus*), which lives only in the elderberry, branches larger than one inch in protected areas should not be pruned or removed from a growing site.[11,17]

Elderberry shrubs typically produce small white flowers (elderflowers) with five petals in the spring, though the elderflowers of the blue elderberry are a creamy yellow color. Small, dark blue-purple berries ripen in the summer in large clusters called umbels or cymes, though there are examples of subspecies that have some variation to these morphologies, such as the blue elderberry that has a white bloom on the berries, causing the berries to look blue, and *S. racemosa*, which are red.[14,17] Variation can also occur within a subspecies due to growing conditions, such as soil type, precipitation, and temperatures, as well as a key differentiation tool: cultivars or genotypes. There are established cultivars or genotypes of the European subspecies (*S. nigra* ssp. *nigra*), such as Sambu or Haschberg, as well as of the American subspecies (*S. nigra* ssp. *canadensis*), like Bob Gordon or Wylewood.[18,19] Cultivars can have more consistent growing patterns, such as blooming or ripening all at once, and desired chemical compositions, such as increased anthocyanins, thus are more desirable to use in large scale growing of elderberry for commercial use.[12] Blue elderberry (*S. nigra* ssp. *cerulea*) does not have any established genotypes to date. If commercial interest in

this subspecies continues to expand, effort should be made to develop cultivars with consistent quality and improved harvestability, which is hampered right now due to flowers and berries ripening throughout a season, instead of a smaller window of time like the American and European subspecies. Indeed, starting this work can help increase the commercial interest viability of the blue elderberry.

Elderberry and Health

A primary driver in interest in the composition of elderberry and elderflower is for their potential health benefits. Several reviews have recently been published on this topic; thus, it will not be explored in depth here. European elderberry has been studied for its antioxidant, antimicrobial, anti-inflammatory, anticancer, immunomodulatory, and antidiabetic properties, as well as neuroprotection and cardiovascular protection *in vitro* and *in vivo*.[10,20,21] These activities have been mainly attributed to the phenolic compounds like cyanidin 3-glucoside and cyanidin 3-sambubioside, but some other compounds have been shown to be bioactive as well, including terpenes, lectins, pectin, peptides, and malic acid.[10,20] Using data from randomized, controlled clinical trials, a recent review found that elderberry could reduce symptoms from upper respiratory viral infections, providing support for the use of elderberry supplements by consumers to combat colds and flus without the use of antibiotic medicine.[22] In a more unique application, elderberry and elderflower extracts have both shown to be effect in combatting gingival inflammation using a topical herbal patch and elderflower tea, respectively.[23,24]

A study of the mechanism of cyanidin 3-glucoside to treat against the influenza virus showed that elderberry extract had some inhibitory effect during the early stages of virus cycle

with stronger impacts during post-infection.[25] The mechanism proposed was that the elderberry extract blocks viral glycoproteins which prevent the virus from attaching or entering cells to replicate, and increases expression of IL-6, IL-8, and TNF. Inflammatory modulating activity of elderberry and elderflower extracts have been investigated.[26] Results showed that quercetin, rutin, and kaempferol are strong inhibitors of nitric oxide production, and metabolites from phenolic degradation including caffeic acid and 3,4-dihydroxyphenylacetic acid were also strong inhibitors without cytotoxicity.

Only a few studies have been done on the bioactivity of *S. nigra* ssp. *canadensis.* In one, the fruit was evaluated for anticancer properties, which showed chemo-preventative activity by inducing quinone reductase and inhibiting cyclooxygenase-2, as well as inhibiting ornithine decarboxylase.[27] These activities are attributed to flavonoids and lipophilic compounds. Another study evaluated two Canadian cultivars of this subspecies evaluated the antiproliferative efficacy of the fruit and flowers on glioma and brain endothelial cells and results showed that elderberry and elderflower extracts inhibited the proliferation of cells under normoxic and hypoxic conditions.[28] The elderberry extracts performed the best and the bioactivities were attributed to the synergistic work of cyanidin 3-sambubioside-5-glucoside and rutin content of the berries, though the rutin concentration in the flowers still had beneficial effects.

Blue elderberry (*S. nigra* ssp. *cerulea*) has only been evaluated as antioxidant activity using the ABTS assay, which indicated that this subspecies has 11.62 ± 0.38 mM Trolox kg^{-1} FW, roughly one third of *S. nigra* ssp. *nigra* evaluated in the same study, where all fruit samples were grown in Slovenia.[29] Further work on elucidating the biological activity of this subspecies through *in vivo* assays and preferably clinical trials should be explored, especially using blue elderberry plants growing in North America to support its use in supplements.

Elderflowers have also been evaluated for their biological activities. A review of antioxidant activity in *S. nigra* ssp. *nigra* flowers has recently been published [2], including ABTS, DPPH, FRAP and CUPRAC assays, therefore it will not be re-summarized here. In general, the data showed that elderflower has higher levels of antioxidant activity compared to the elderberry. Similarly, elderflower extracts had higher nitric oxide inhibition compared to elderberry extracts *in vitro.*[26] Elderflower is antidiabetic by increasing insulin-dependent glucose uptake, diuretic, treat respiratory infections, antiviral.[10] While phenolic compounds like flavonols are presumed to be the most active compounds, pectic polysaccharides are also bioactive in elderberry and elderflower, inducing complement fixing and macrophage stimulation.[30–32]

Flowers of the blue elderberry have been evaluated for their antioxidant activity using the ABTS assay, which showed they have 44.87 ± 0.54 mM Trolox kg^{-1} DW, significantly less than flowers of *S. nigra* ssp. *nigra* (118.26 ± 3.10 mM Trolox kg^{-1} DW). Aqueous extracts of wild elderflowers of this subspecies were also found to have neuroprotective effects, especially related to Parkinson's disease, by increasing the antioxidant response mediated by Nrf2 in cortical astrocytes and improving mitochondrial function in neurons.[13] Unfortunately, that study did not include any growing information about the elderflowers or the concentration of the phenolic compounds in the extract, which would have helped other researchers replicate and expand on the results.

While there have been promising studies on the impact of elderberry and elderflower extracts to combat illness and disease, more *in vivo* studies and clinical trials should be performed to better understand the mechanisms of the bioactivity as well as to determine which compounds are responsible for the bioactivity, particularly in the lesser-known subspecies *canadensis* and

cerulea. This can better inform people involved with the cultivation of elderberry to select for varieties that have the compounds of interest.

Elderberry Applications and Market

The market for herbal supplements has been growing in the recent decade and immune system-supporting supplements had a huge spike in sales during the COVID-19 pandemic. Elderberry products are a popular option of alternative medicine in hopes of improving and protecting health.[33] Beverages are a popular use of the elderberry, including syrup or other tonics made by soaking the berries in water or alcohol. It can also be found as an ingredient in various kombuchas, juices, energy drinks, wine, and tea. Elderberry is typically mixed with a variety of other ingredients, including but not limited to ginger, honey, echinacea, and other spices. [12] More recent products using elderberry include gummies typically marketed as health supplements, lozenges, tablets, and powdered berries especially as part of a drink mix. Elderberries are also frequently used in jams and jellies. Pomace, the byproduct of juicing, has been studied for its benefits when incorporated into other products just as baked goods.[34]

Beyond its potential for bioactive products to benefit consumers, elderberry can also be used as a natural food dye due to the high concentration of anthocyanins [35], which can be used in place of artificial red or purple food dyes, particularly in acidic foods. Its application in edible films has recently been investigated, explored various biopolymers that could retain the phenolic compounds of elderberry in the film so that they can remain active to protect foods.[36] Active edible films can be an effective solution to reduce plastic packaging and food waste due to spoilage.

Cosmetic and skin care applications are also an area of interest, [37] and current products on the market that include elderberry include lip color, toner, face mask, and Epsom salt.

Elderberry Composition

Future chapters will focus on evaluating the blue elderberry and elderflower for their composition. Herein, the data available on the other elderberry subspecies of interest (European and American elderberry) are summarized to provide a basis of the expected composition as well as information to compare the subspecies for their composition.

Elderberries have a high amount of water, at about 80%.[2,38–40] The main sugars in elderberry are glucose and fructose, with some small amounts of sucrose.[8,29,41] Sorbitol was also measured, which was very minor compared to the other three sugars and was seen in the highest concentrations in the wild elderberry.[42] Citric acid is the main organic acid in elderberry, with malic acid the next highest acid. Small amounts of shikimic, tartaric, and fumaric acid have been measured in elderberry as well. [8,29,41]

Only data on European elderberry is available for microconstituents such as vitamins, minerals, fatty acids, and amino acids. Vitamins found in elderberry include various B vitamins, vitamin C, and vitamin E.[10,43,44] The main minerals are magnesium, calcium, and potassium.[45,46] Because studies of these micronutrients have only been performed on the European elderberry, it is important for further work to include other subspecies, including the American and blue elderberry so that better comparisons can be made.

Phenolic Content

An important group of bioactive compounds found in fruit and vegetables is phenolic compounds, which consist of one or more phenolic groups (benzene ring with a hydroxyl group). Types of phenolic compounds include phenolic acids and flavonoids; flavonoids can be further separated into groups such as anthocyanins, flavonols, flavan-3-ols, and flavones. Phenolic compounds may have some biological activity, although bioavailability can be very low.[47]

A common, albeit imperfect, way to measure phenolic content of elderberries is using a colorimetric method like Folin-Ciocalteu which can measure a complex that forms between phenolic compounds and molybdenum-tungsten at 765 nm.[48] Because this method measures all reducing agents in the matrix, reducing sugars and ascorbic acid will also react and increase the absorption thus inflating the total phenolic content (TPC). Standard curves are typically constructed using gallic acid, hence the units for TPC are gallic acid equivalents (GAE).

TPC (in mg GAE per 100 g) in European elderberries can vary greatly but reported values include 461 ± 121 [49] and 683 ± 49.[29] In American elderberry, TPC has been reported to be 390 ± 56 [50], 593 ± 70.[18] One study has included blue elderberry grown in Slovenia, which had a TPC of 416 ± 31.[29] However, because of the imprecise nature of this assay, it is important to identify and measure the concentration of each phenolic compound present whenever possible, the results of which is explored in the following sections.

Anthocyanins

Anthocyanins are water-soluble pigments in plants, and they give elderberries their blue-purple hue.[51] Total monomeric anthocyanin content (TMA) is typically measured using the pH differential method, which takes advantage of the change in light absorption of anthocyanins in

solutions with different pH and the unit is typically cyanidin glucoside equivalents (CGE).[51] European TMA content of European elderberry can range from 170 ± 12 to 343 ± 11 mg CGE 100 g^{-1} FW.[49] In American elderberry, the range of TMA content is 354 ± 59 to 595 ± 26 mg CGE 100 g^{-1} FW [52] and 106 ± 2 to 444 ± 14 mg CGE 100 g^{-1} FW.[49]

Analysis of the phenolic compounds via high performance liquid chromatography with UV-Visible light detection (HPLC-UV/Vis) or with mass spectrometry (HPLC-MS) have elucidated a variety of molecules present in the European elderberry. Anthocyanins, a type of flavonoid and popular for their red to blue pigments, are of high interest in elderberry. Most studies have found that cyanidin (cyn)-based anthocyanins are the dominant type in European and American elderberry, including cyn 3-*O*-sambubioside (3-*O*-(2-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside) and cyn 3-*O*-glucoside.[1,8] Cyn 3-sambubioside-5-glucoside and cyn 3,5-diglucoside are also commonly seen in the elderberry.[1,53]

The American elderberry (*S. nigra* ssp. *canadensis*) has a more unique anthocyanin profile with high presence of acylated anthocyanins compared to the European elderberry, including cyn 3-*O*-coumaroyl-sambubioside-5-*O*-glucoside (the most concentrated anthocyanin), cyn 3-*O*-coumoaryl-sambubioside.[54] These acylated anthocyanins may be more stable during processing, but the authors found that cyn 3-*O*-coumaroyl-sambubioside was the least stable anthocyanin during storage (<38% of content after 3 months of frozen storage), whereas cyn 3-*O*-cou-sam-5-*O*-glu and cyn-3-*O*-sam-5-*O*-glu were more stable.[55]

Flavonols, phenolic acids, and other phenolic compounds

Another major type of phenolic compound in elderberry is flavonol glycosides, which include rutin (quercetin 3-*O*-rutinoside), isorhamnetin 3-*O*-glucoside or 3-*O*-rutinoside, and kaempferol 3-*O*-rutinoside. Rutin has frequently seen to be the most concentrated flavonol in European elderberry, and often the most concentrated phenolic compound of any present.[8] *S. nigra* ssp. *canadensis* also contains higher levels of rutin than other flavonols.[49,54,56] Other flavonol glycosides present in elderberry include kaempferol and isorhamnetin derivates, such as kaempferol-rutinoside, isorhamnetin-rutinoside, and isorhamnetin-glucoside.[1,49,54] Phenolic acids are also present in high amounts in elderberry, including chlorogenic acid isomers (caffeoylquinic acid), *p*-coumaric acid, sinnapic acid, cinnamic acid, and ferulic acid.[57] Flavan-3-ols found in elderberry include (+)-catechin, (-)-epicatechin, and procyanidins.[58,59]

Cyanogenic Glycosides

Parts of the elderberry plant are known for having toxic compounds called cyanogenic glycosides (CNGs) that, if consumed, can be dangerous due to the release of cyanide. The stems and leaves have the highest concentration of CNGs, followed by unripe berries and flowers, followed by ripe berries and cooked juices.[1,6,14,60] The primary CNG in elderberry is sambunigrin, which is a diastereoisomer of the more commonly known CNG prunisin. Amygdalin is the next most common CNG, though it is not often measured. Dhurrin and linamarin have also been measured in elderberry plant material.

European elderberry levels of CNGs can vary greatly depending on the growing location, such that concentrations ranged from 0.08 concentrations ranged from 0.08 ± 0.01 to 0.77 ± 0.08 μg g^{-1} when fruit was evaluated from various altitudes in Slovenia.[6] These concentrations are lower

than those detected in elderberry juice, found to be 18.8 ± 4.3 mg kg^{-1} in raw juice and 10.6 ± 0.7 mg kg^{-1} in cooked elderberry juice, suggesting that thermal processing can reduce CNG levels in elderberry products.

American elderberries have been evaluated for their concentrations of CNGs.[60] These include amygdalin, sambunigrin (indistinguishable from isomer prunasin in analysis), linamarin, and dhurrin. Specifically, the Ozone and Ozark genotypes were evaluated, giving better insight into how CNG concentrations may be impacted by plant genetics. While the total concentrations of the four CNGs in the two American elderberry genotypes were somewhat similar (4.01 µg g^{-1} in Ozone and 3.66 µg g^{-1} in Ozark), the composition of which CNGs made up that total were quite different: Ozone elderberries had similar levels of amygdalin and sambunigrin (1.57 and 1.45 µg g^{-1} respectively) while Ozark elderberries had much higher levels of amygdalin than sambunigrin (2.36 and 0.36 µg g^{-1} respectively).

Volatile Compounds

The flavor profile of elderberries is an important factor in the consumer sensory experience with elderberry products. Two of the most common compounds identified as drivers of elderberry aroma identified in multiple studies of the berries or elderberry juice are β-damascenone and dihydroedulan.[19,61–66] Nonanol was also identified as a key volatile compound contributing to the characteristic elderberry aroma [63], while ethyl-9-decenoate was found to be important for the characteristic elderberry aroma by another study [67]. While these volatile compounds can be key to the unique aroma, they are not typically the most concentrated compounds. Studies have found the most concentrated compounds to be linalyl acetate, linalool, [64] phenylacetaldehyde, benzaldehyde

[61,63], hexanal, 2- and 3-methyl-1-butanol, nonanal and benzaldehyde[62]. However, comparing concentration of compounds across studies can be difficult due to differences in sample preparation, extraction method, and method parameters, to name a few important factors.

Neither American nor blue elderberry has been evaluated for their volatile aroma composition, which limits the understanding of how these subspecies may perform and be accepted by consumers in the same formats as European elderberry. Analytical assessments of the elderberries and products using the elderberries, in addition to sensory panels (descriptive and/or acceptance) would be useful information for product developers and should be performed when cultivars or genotypes are being selected for cultivation and use in commercial products.

Elderflower Applications

Elderflowers are frequently used in beverages and food products, including but not limited to teas, syrups, lemonades, liqueurs, wines, jams/marmalades, and tonic water. They are also used for flavoring in yogurt, coated almonds, lozenges, and confectionary goods, to name a few. Furthermore, elderflower can now commonly be found in soaps, lotions, and candles, thus consumers, especially in the United States are becoming more familiar with elderflowers, which have been well-known in Europe for generations. Topical applications are also being explored for their benefits to skin.[68,69] These recent studies support the long history of use of elderflower by the Lumbee tribe in North Carolina, who use elderflower as a treatment for skin cancer by soaking flowers in witch hazel for a week then applying that to the skin.[13]

Elderflower Composition

The main compound in elderflowers, like elderberries, is water, and is found in similar concentrations (about 75%).[70] Glucose, fructose, and sucrose make up the main sugars found in elderflower.[71] While European elderflowers have a roughly equal amount of these sugars, elderflowers of the blue elderberry have a much higher level of fructose than glucose or sucrose. However, there has only been one study to measure these compounds in elderflowers, and more studies are needed to know if this trend occurs across each of the subspecies. There is limited data on these compounds across the three subspecies of interest, such as no information on the American elderberry; thus, few comparisons can be made.

Minerals and vitamins have been evaluated in European elderflowers. Minerals include calcium, magnesium, copper, zinc, and manganese.[72] Calcium is the most concentrated mineral with an average of 2955.9 ± 272.7 μg g^{-1} across several wild and cultivated samples and magnesium is the next most concentrate mineral at an average of 1200.2 ± 453.6 μg g^{-1}.[72] Vitamin C has only been measured in elderflower syrup, ranging from 22.47 ± 0.06 mg L^{-1} to 46.17 mg L^{-1} .[71,73]

Phenolic Compounds

Elderflowers of the European subspecies have been evaluated several times for their phenolic profile. Dominant compounds in the flavonol rutin and neochlorogenic acid. Concentrations can vary greatly, just like many of the other compounds already explored in this review. Growing and harvest conditions[6] or extraction parameters[71] can impact the final concentrations reported. Significant differences in phenolic concentrations have been found between cultivars, such that the concentration of rutin ranged from 11.6 to 42.3 mg g^{-1} dry weight

and neochlorogenic acid ranged from 10.1 to 20.7 mg g^{-1} dry weight among the 16 genotypes.[74] The coefficient of variation was greater than 10% for all of the compounds measured, including nine phenolic acids and six flavonol glycosides.

American elderflowers have also been studied for their concentration of rutin and chlorogenic acid which generally align with the European elderflower profile, except that the primary phenolic acid was chlorogenic acid instead of neochlorogenic acid.[56] American elderflower appears to contain a different chlorogenic acid isomer than the European elderflower, which has mainly neochlorogenic acid. Furthermore, 12 cultivars were sampled for the study, which showed high variability in concentration of the two compounds measured. Rutin concentrations ranged from 4637 to 8111 mg kg^{-1} while chlorogenic acid concentrations ranged from 1180 to 3064 mg kg^{-1}, showing that key phenolic compounds can be more than double in some cultivars. The concentration of these two compounds did not appear correlated, as the correlation coefficient was only 0.018.

Cyanogenic Glycosides

While there have been several studies measuring the CG content in elderberries of different subspecies, the data available on elderflower CG content is limited. In fact, only one study has published data on this area to date and it focused on European elderflowers. A study comparing growing locations at multiple altitude levels (from 210 to 1077 meters above sea level) to determine impact on phenolic compounds and cyanogenic glycosides found that CG concentrations in elderflowers ranged from 1.23 ug g^{-1} to 18.88 ug g^{-1}, generally increasing as the

altitude increases.[6] Sambunigrin was the only CG measured and compared to the berries of the same plants in this study, elderflowers contained more CGs than elderberries.[6]

Elderflowers from the American subspecies nor the blue subspecies have been analyzed for their CG content. As consumer concern for this toxic group of compounds remains high, it would increase confidence of consumers to utilize the elderflowers of these other subspecies if data was available on the CG concentrations of these flowers.

Volatile Compounds

Elderflowers and elderflower products (i.e., syrups and essential oils) have been investigated for their volatile profile. A direct comparison is difficult to make from the syrups, which have other ingredients like sugar or lemons, to the plain flower extracts, but due to the high popularity of elderflower syrups, the results of those studies are included here as well.

In studies of the European elderflower without any additional food ingredients, linalool and linalool derivates, such as (*Z*)-linalool pyranoxide and *cis*-linalool oxide, have frequently been identified as prominent.[75–79] The aroma of linalool, the main aroma compound in lavender, can be described as citrus, fruity, floral, and woody.[80] The age of the flower when harvested as well as how the flowers are stored after harvest (such as different drying and freezing techniques) can greatly impact the volatile profile.[76,77] As expected from the other data on inter-cultivar variation, the volatile profile is heavily influenced by the cultivar.[81] For example, wild elderflower had twice as much rose oxide and more linalool oxide than the other 12 cultivars.[81] While this could be a challenge for manufacturers that use elderflower in products to have a consistent aroma from batch to batch, it also allows for more selectivity to find a cultivar that matches desired organoleptic

properties in the product. American elderflowers have not yet been evaluated for their volatile profile, nor have blue elderflowers.

Conclusions

Elderberry and elderflower are becoming more common ingredients and flavoring agents in beverages and food products. However, a vast majority of the products on the market today utilize the European subspecies of this plant due its more established cultivation and a deeper understand of the composition, particularly the phenolic profile, of the fruit and flower. North American subspecies, the American elderberry *S. nigra* ssp. *canadensis* and the blue elderberry *S. nigra* ssp. *cerulea*, have some information available regarding composition, but further analyses are needed to understand how they may perform in the common applications that European elderberry and elderflower are used in today.

Chapters 1 and 2 have been published in *ACS Food Science & Technology*. Chapter 3 is a drafted manuscript and will be submitted for publication.

Chapter 1. Blue elderberry (*Sambucus nigra* ssp. *cerulea*): a robust and underutilized fruit for value-added products

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Abstract

Blue elderberry (*Sambucus nigra* ssp. *cerulea*), a subspecies native to the western United States, is frequently planted in farm hedgerows in California for their ecological benefits and they thrive in challenging environments produced by climate change. Key properties of blue elderberry were evaluated for the first time over two growing seasons to better understand how this subspecies compares to those typically used in food, beverages, and supplements. The soluble solids, titratable acidity, and pH of the blue elderberry were similar to common elderberry subspecies (*S. nigra* ssp. *nigra* and *S. nigra* ssp. *canadensis)*. Total monomeric anthocyanin levels were about half to a quarter of the level compared to other subspecies, whereas total phenolic content was similar to that of other elderberry subspecies. Higher levels of flavonols (i.e., rutin and isorhamnetin-rutinoside) were found in the blue elderberry compared to other subspecies. Two phenolic compounds, 5-hydroxypyrogallol hexoside and protocatechuic acid dihexoside, were tentatively identified in the blue elderberry using accurate mass QTOF-MS/MS and are unique to this subspecies. Data demonstrate that this subspecies grown in California can be used in for food and beverage applications like other elderberry subspecies.

Keywords: Food composition, Anthocyanins, Phenolic content, Food analysis, Berries, Fire-resilient

1. Introduction

As global warming and water scarcity issues continue to impact food systems, fire-resilient and drought-tolerant plants will become more important for supplying nutrient-rich foods [82]. Wildfires throughout the western United States are becoming more common and more serious as seasons are hotter and drier. California has been experiencing unprecedented levels of wildfires, including over 1.9 million acres burned in 2018, over 4.2 million acres burned in 2020, and over 2.5 million acres in 2021 [83]. One native and fire-resilient plant is the blue elderberry (*Sambucus nigra* ssp. *cerulea*), which grows wild throughout the western United States and has become a popular choice to grow in hedgerows. The blue elderberry is drought-tolerant, and the roots of the blue elderberry can survive fires to regrow the next season to continue providing valuable flowers and fruit [84,85], making it an ideal choice to plant in regions of California and American West often stricken by wildfires.

While European (*S. nigra* ssp. *nigra*) and American (*S. nigra* ssp. *canadensis*) elderberries have been studied for decades, there is currently little information on the subspecies native to the western region of North America, *S. nigra* ssp. *cerulea* (syn *caerulea*, *Sambucus mexicana*), known as blue elderberry due to a white-colored bloom on the exterior of the berry which makes it appear blue. In California, it grows wild in riparian ecosystems near rivers and streams [86], but is also planted in hedgerows (i.e., rows of shrubs, grasses, and trees planted on the borders of agricultural fields, streams, and irrigation ditches) on farms to improve water, air, and soil quality, in addition to providing a habitat for birds, pollinators, and other beneficial insects [87]. The plant can grow several meters tall and wide and flowers from May to August, with peak fruit ripening throughout July and August. While elderberry prefer moist soil and some hedgerows may receive some irrigation during the summer months, most are not irrigated once the hedgerow has been

established, about 2-4 years [84]. That is one of the benefits of using native and drought-tolerant plants, as they can better withstand the natural climate without excess resources.

Elderberries have a long history of use by Native Americans and Europeans in foods, beverages, and herbal medicines. Research exploring links between elderberry consumption and health has increased dramatically, particularly in the past decade. Numerous *in vitro* and *in vivo* studies demonstrate that elderberries have potent antioxidant, antibacterial, and antiviral properties [88–92]. Results of two randomized, double-blind, placebo-controlled clinical trials suggest that elderberry supplements reduce the duration and severity of cold symptoms [91,92]. Roscheck et al. (2009)[88] identified two non-anthocyanin flavonoids in elderberry extract that inhibited viral ability to infect host cells when bound. While most bioactivity of elderberries is assumed to result from the phenolic compounds like anthocyanins [93], the high-molecular weight fraction of concentrated elderberry juice was found to contain acidic polysaccharides that had potent effects against the human influenza virus [94]. The health-promoting properties of elderberry have led to recent increases in its use in products such as supplements, syrups, gummies, and teas, as well as wine and jams. During the COVID-19 pandemic, elderberry supplements gained wide attention because of potential anti-viral activities; however, there is no strong clinical evidence that elderberry could be beneficial in preventing or treating COVID-19.

The market for elderberries is expected to continue to increase, as the sales of herbal dietary supplements was over $11 billion in 2020, a 17.3% increase from 2019. Elderberry was the top-selling herbal supplement, with sales over $275 million, as consumers became more interested with supporting their immune systems [9]. In addition to the interest in elderberry as an ingredient in functional foods, elderberry can be an excellent source of natural coloring agents for food and beverage applications due to the high content of red and purple anthocyanins [35].

Characterization of the chemical composition, functional properties, and impact of processing on the bioactive compounds in elderberry is largely limited to *S. nigra* ssp. *nigra* and, to a lesser extent, *S. nigra* ssp. *canadensis*. *S. nigra* ssp. *nigra* is commonly referred to as the European black elderberry, which has many established cultivars, such as "Haschberg" and "Samyl", and it has an established market. The European elderberry is the most frequently used subspecies in commercial elderberry-based products and has been extensively studied for its composition [8,29,95], anthocyanin stability [96,97], and health benefits in European black elderberry-based products [6,98]. *S. nigra* ssp. *canadensis* is commonly referred to as the American elderberry, a subspecies native to the eastern and central regions of North America. There are several cultivars of the American elderberry, including "Johns" and "Bob Gordon". The American elderberry, which is utilized in small-batch products, has also been evaluated for its composition [49,50,52,54] and health-promoting properties [99]. The acreage grown of this subspecies has been increasing rapidly and there is a goal to grow over 2,000 acres by 2025, according to the Midwest Elderberry Cooperative.

Currently, there is no information on the chemical composition of the fruit of the blue elderberry (*S. nigra* ssp. *cerulea*). With the recent increase in demand for elderberry, blue elderberry grown in hedgerows may be an additional and valuable source of bioactive phenolics and natural colorants. The objective of this study was to determine the moisture content, soluble solids, pH, titratable acidity, and establish the anthocyanin and phenolic profiles of blue elderberries grown in Northern California to support the use of this robust, native crop in commercial products.

2. Materials and methods

2.1 Plant Material

Hedgerows of *S. nigra* ssp. *cerulea* were identified on five farms near Davis, California in Spring 2018 with the assistance of an experienced agronomist at The Cloverleaf Farm (Dixon, CA). Farm, hedgerow, and harvest information is presented in Table 1. Blue elderberries were determined to be ripe when the berries in a cyme (cluster) were deep purple, with or without the white bloom, and had no green berries present. Ripe elderberries were harvested by hand from all four quadrants of the elderberry shrub, totaling approximately 3 kg of elderberries. The berries were placed in clear plastic bags, stored on ice, and transported to the laboratory. A subsample (about 5 g) was separated for moisture analysis, while the rest was de-stemmed and stored at -20 °C until analyzed.

Table 1. Farm information, including their coordinates and altitude

farm ID	1	2	3	4	5
farm name	The Cloverleaf Farm, Dixon, CA	Center for Land-based Learning, Winters, CA	Citrona Farms, Winters, CA	Pacific Star Gardens, Woodland, CA	Lockewood Acres, Vacaville, CA
GPS coordinates	38.513054, -121.784400	38.523090, -121.916133	38.630538, -122.011418	38.635551, -121.785811	38.442719, -121.948788
altitude (m)	17.0	34.0	71.0	20.0	37.0
hedgerow ID (year planted)	1 (2014), 2 (2018)	3 (2000), 4 (2006), 5 (2008)	6 (1996), 7 (1998), 8 (2006), 9 (2008), 10 (2015)	11 (1997), 12 (2009), 13 (2012)	14 (2018)
date of 2018 harvest	07-23-2018, [a]	07-26-2018	07-13-2018	07-20-2018	not harvested
date of 2019 harvest	07-24-2019, [a] 08-05-2019 [b]	07-18-2018	07-12-2019	07-25-2019	08-08-2019

[a] date of harvest for hedgerow 1. [b] date of harvest for hedgerow 2

2.2 Chemicals

HPLC grade methanol (MeOH), acetonitrile (ACN), phosphoric acid, ethanol, hydrochloric acid, and sodium hydroxide (NaOH) were purchased from Fisher Scientific (Fair Lawn, NJ). Ascorbic acid was purchased from Acros Organics (Fair Lawn, NJ). Formic acid, gallic acid, sucrose, chlorogenic acid, rutin, and catechin, and Folin-Ciocalteu reagent were purchased from Sigma Aldrich (St. Louis, MO). Cyanidin-3-glucoside was purchased from Extrasynthese (Genay Cedex, France). Ultra-pure water was obtained from a Milli-Q water system (Millipore Sigma, Burlington, MA).

2.3 Determination of water content

To determine water content in elderberries, 2 g of fresh elderberries were weighed on aluminum pans and placed in a 70 °C oven until the measured mass was consistent over three time points. Duplicate samples were prepared from each shrub and averaged. Water content was calculated using the equation below:

$$Water\ content\ (\%) = \frac{Fresh\ mass\ (g) - Dry\ mass(g)}{Fresh\ mass\ (g)}\ x\ 100\%$$

2.4 Sample Preparation

From each shrub, about 250 g of frozen berries were thawed in a glass container overnight at 4 °C. The following day, the thawed berries were mashed for 4 min by hand using a plastic pestle, then homogenized (Ultra Turaxx T18, IKA Works, Wilmington, NC), and centrifuged (Sorval ST16, Thermo Fischer Scientific, Waltham, MA) at 3,000 rpm (1690 G) for 7 min. The supernatant was strained, collected, and weighed. A 15 mL aliquot of sample extract was stored in a 15 mL plastic tube at -20 °C for total monomeric anthocyanin (TMA) analysis. The remaining supernatant

was used to determine soluble solids, pH, and titratable acidity (TA). One pooled sample was analyzed from each shrub and analyzed in duplicate analytical repetitions.

2.5 Sample analysis

2.5.1 Determination of soluble solids, pH, and TA

A refractometer (Abbe Mark III, Reichart Technologies Analytical Instruments, Buffalo, NY) was used to determine soluble solids. It was calibrated with standard solutions of 5°, 10°, and 15° Brix made with sucrose and water. A 150 µL aliquot of elderberry juice was placed on the prism and read using the automatic setting. The pH was determined using a SevenMulti pH meter (Mettler Toledo, Columbus, OH). It was calibrated before each use using buffers at pH 4.0, 7.0, and 10.0. To determine TA, 10 mL of elderberry juice was diluted to 100 ml with nanopure water and mixed. This dilute juice was titrated to pH 8.2 using 0.1 N NaOH. The volume of 0.1 N NaOH used to achieve the desired pH was used to calculate the mg citric acid per 100 g fresh weight (FW). For each of these analyses, duplicates were run on each juice.

2.5.2 Determination of total monomeric anthocyanin content (TMA)

TMA was quantified using the pH differential method (AOAC 2005.02) [51]. Juice was diluted 1:50 with the buffer (0.025 M potassium chloride at pH 1.0 and 0.4 M sodium acetate at pH 4.5). Absorbance was read at 520 and 700 nm using a UV visible spectrophotometer (Shimadzu, Kyoto, Japan). Duplicate analyses of each juice were analyzed. Cyanidin-3-glucoside was used for quantitation parameters, using molecular mass of 449.2 Da and extinction coefficient (ε) = 26,900 L (cm mol)$^{-1}$. Results are expressed as mg cyanidin-3-glucoside equivalents (CGE) per 100 g FW.

2.5.3 Determination of total phenolic content (TPC)

Elderberries were extracted by combining 5 g frozen berries with 25 mL MeOH:formic acid (97:3, v/v) in a conical tube. The contents were homogenized, placed in a shaker without water (Gyrotory water bath shaker, New Brunswick Scientific Co. Edison, New Jersey) at speed 7.5 for 20 min, then centrifuged at 3,000 rpm for 7 min. The supernatant was transferred to a 15 mL plastic tube and stored at -80 °C for no more than two weeks prior to analysis. Duplicate extracts were made from each shrub. TPC was determined using the Folin-Ciocalteu method. First, elderberry phenolic extract was diluted 1:4 with water. Each extract was analyzed in duplicate and averaged. In 10 mL glass tubes, 6 mL water was combined with 100 µL sample (dilute elderberry extract or gallic acid standard, 50-600 mg/L, or water as a blank) and 500 µL Folin-Ciocalteu reagent. After mixing and incubating for 8 min at room temperature, 1.5 mL 20% (w/v) aqueous sodium carbonate was added. The tubes were mixed, covered with foil to avoid light exposure, placed in a water bath at 40 °C for 40 min, then cooled at room temperature for 15 min. The samples were read by a UV visible spectrophotometer at 765 nm and quantified using an external standard curve prepared with gallic acid ($R^2 \geq 0.99$). TPC is expressed as mg gallic acid equivalents (GAE) per 100 g FW.

2.5.4 Relative quantitation of phenolic compounds via HPLC-DAD-FLD

Five grams of frozen berries were mixed with 25 mL of (50:50 water:ethanol v/v with 0.1% HCl and 0.1% ascorbic acid) in a conical tube, which was then homogenized for 1 min at 7,000 rpm . The mixture was stored at 4 °C overnight, then in the morning, centrifuged at 4,000 rpm for 7 min. The supernatant was used directly for analysis. Three pooled samples were made for each hedgerow, each consisting of even amounts (by mass) of berries from three distinct shrubs. Each

pooled sample was extracted once to give 3 biological replicates, and each extract was run in duplicate (analytical replicates). Averages concentrations for compounds were determined across the hedgerow in mg per 100 g FW. The concentration of phenolic compounds in blue elderberry followed the method by Giardello et al. (2020) with some modifications. Briefly, samples were analyzed via reversed-phase liquid chromatography (RP-HPLC) on an Agilent 1200 with a diode array detector (DAD) and fluorescence detector (FLD). The column used was a PLRP-S 100A 3 µm 150 x 4.6 mm (Agilent Technologies, Santa Clara, CA, USA) at 35 °C, and the injection volume was 10.0 µl. Mobile phase A was water with 1.5 % (v/v) phosphoric acid, while mobile phase B was 80%/20% (v/v) acetonitrile/ mobile phase A. The gradient used was 0 min 6% B, 73 to 83 min 31% B, 90 to 105 min 6% B. The DAD was used to monitor hydroxybenzoic acids at 280 nm, hydroxycinnamic acids at 320 nm, flavonols at 360 nm, and anthocyanins at 520 nm. The FLD was used to monitor flavan-3-ols, with excitation at 230 nm and emission at 321 nm. External calibration curves were prepared using chlorogenic acid for phenolic acids, rutin for flavonols, and cyanidin-3-glucoside for anthocyanins , at the following concentrations: 200, 150, 100, 75, 50, 25, 10, 5, and 2.5 mg/L. Catechin was used to quantify flavan-3-ols and standards were run at 150, 100, 75, 50, 25, 10, 5, 2.5, and 1 mg/L. Compounds were identified based on retention time and spectral comparisons with standards. Information about the linear equations and lower limits of detection (LLOD) and quantitation (LLOQ) can be found in Table S1 in the supplementary material. The LLOD was calculated as 3.3 times the standard deviation of the y-intercept of the curve divided the slope, while the LLOQ was calculated as 10 times those values.

2.5.5 Identification of phenolic compounds by high pressure liquid chromatography (HPLC) and quadrapole time of flight tandem mass spectrometry (QTOF-MS/MS).

Several peaks appeared in the HPLC chromatograms that could not be identified using the above parameters. Chromatographic eluents of these peaks were collected individually and dried under vacuum. These extracts were reconstituted with mobile phase A, and 5 µL were injected into the HPLC- QTOF-MS/MS for accurate mass analysis (Agilent 1290 Infinity II HPLC coupled to a 6545 QTOF).

A Poroshell 120 EC-C18 column (Agilent, Santa Clara, USA) was used (2.1 x 150 mm, 2.7 µm) at 35 °C. Mobile phase A was 1% formic acid in distilled water, and mobile phase B was 1% formic acid in acetonitrile. The gradient used was 0 min 3% B, 30 min 50% B, 31-32 min 95% B, 33-38 min 3% B. The mass spectrometer was used in negative mode, and the mass range for MS was 100 to 1000 m/z while the range for MS/MS was 20-700 m/z. Collision energies at 10, 20, and 40 V were applied. The drying gas was set to a flow of 12 L/min at 250 °C, while the sheath gas was set to 11 L/min at 350 °C. The nebulizer was set to 40 psig, the capillary voltage was 3500 V, the nozzle was set to 500 V, and the fragmentor was set to 100 V. Data was analyzed using Agilent MassHunter Workstation Qualitative Analysis 10.0 (Agilent, Santa Clara, CA, USA). Tentative identification was achieved by comparing the mass to charge ratio of the precursor and fragment ions to online libraries of compounds as well as using formula generation for the peaks in the spectra.

2.6 Statistical analysis

Data was analyzed using R software version 3.6.2. (R Core Team, 2019). Differences between harvest years was analyzed by Student's t-test ($p < 0.05$). Differences between hedgerows and farms was analyzed using two-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons ($p < 0.05$) at a confidence level of 95%.

3. Results and Discussion

3.1 Blue elderberry macronutrient composition

The composition of blue elderberries (*S. nigra* ssp. *cerulea)* is presented for the first time, which is key to understanding how this subspecies of *Sambucus nigra* compares to commercialized elderberry subspecies, *S. nigra* ssp. *nigra* and *S. nigra* ssp. *canadensis*. These data help to establish the blue elderberry grown in hedgerows in California as a viable source of berries and bioactive compounds. Data for the compositional assays is presented for the 2018 and 2019 harvest years as the average of all shrubs sampled in Table 2. The average moisture for the blue elderberries was 79.5 ± 1.5% in 2018 and 79.5 ± 1.6% in 2019, which is very similar to the levels found in wild elderberries in Spain (78.91 ± 0.927 g per 100 g FW) [95]. The average soluble solids found in blue elderberry ranged from 11.94 ± 2.08 to 14.95 ± 1.02 g per 100 g FW in 2018 and from 12.64 ± 1.86 to 17.09 ± 1.60 g per 100 g FW in 2019. These values are slightly higher than the soluble solids found in *S. nigra* ssp. *cerulea* grown in Slovenia[29] and American elderberries grown in Ohio[52]. Compared to European and American elderberries evaluated in other studies, blue elderberries have similar levels of soluble solids [8,18,29,49,50,95]. In the present study, the overall average content of soluble solids was significantly different ($p < 0.01$) between years, as blue elderberries harvested in 2019 had significantly higher average soluble solids (14.90 ± 1.26 g per 100 g FW) than the elderberries harvested in 2018 (13.57 ± 0.48 g per 100 g FW). The pH in the blue elderberry ranged from 3.44 to 3.86 in 2018 and from 3.46 to 3.79 in 2019, with no significant difference found between harvest years. These values are slightly lower than the values found in European elderberry, which ranged from 3.9 ± 0.06 to 4.1 ± 0.04 with an average pH of 3.9 ± 0.2, and American elderberry, which ranged from 3.9 ± 0.04 to 4.5 ± 0.03 with an average pH of 4.2 ± 0.2 [49] Another evaluation of pH in American elderberries had a range of 4.5 ± 0.08 to 4.9 ±0.12,

higher than those found in the blue elderberry.[52] The higher sugar and lower pH levels in blue elderberry could potentially impact taste and performance in food and beverages as compared with the European and American species. The average titratable acidity in blue elderberries ranged from 0.45 ± 0.08 to 0.77 ± 0.03 g citric acid per 100 g FW in 2018 and from 0.54 ± 0.06 to 0.77 ± 0.11 g citric acid per 100 g FW in 2019 with no significant difference found between harvest years. These values are lower than the total acids found by Mikulic-Petkovsek et al. (2016)[29] in *S. nigra* ssp. *cerulea* (1.63 ± 0.02), but they are similar to the levels found in European elderberry [8,18,49,50].

Table 2. Average, standard error, and sample size for the compositional assays performed on California blue elderberries.

	2018			2019		
	mean ± SE	RSD (%)	n	mean ± SE	RSD (%)	n
Water content [a]	79.5 ± 1.5 a	1.9	63	79.5 ± 1.6 a	2.0	77
Soluble solids [b]	13.57 ± 0.48 b	3.54	63	14.90 ± 1.26 a	8.46	68
pH	3.66 ± 0.08 a	2.2	63	3.66 ± 0.10 a	2.7	68
TA [c]	0.60 ± 0.10 a	16.7	63	0.65 ± 0.07 a	10.8	68

[a] percentage (%). [b] g per 100 g FW. [c] g citric acid per 100 g FW. Significance tested by Student's t-test, letters that differ between values in a row indicate significant difference ($p < 0.05$).

3.2 Total monomeric anthocyanins

Anthocyanins are a class of phenolics that contribute red, purple, and blue hues to fruits and vegetables, act as attractants for pollinators, and are potent antioxidants. European and American elderberries are well-known for containing high levels of anthocyanins [8,18,49]. The anthocyanin content of elderberries strongly correlates to the antioxidant potential of the fruit, which may confer health-promoting properties [50,89], which is one reason why elderberries are used in supplements and value-added products. Elderberry is also used as a source of natural food colorants due to the levels of anthocyanins[35]. Understanding the levels of anthocyanins in the blue

elderberry grown in hedgerows is critical towards establishing this native fruit as an additional and more sustainable elderberry. The average TMA measured in blue elderberry ranged from 34.2 ± 9.7 to 113.4 ± 18.2 mg CGE per 100 g FW in 2018 and from 43.1 ± 11.5 to 121.5 ± 11.5 mg CGE per 100 g FW in 2019 (Table 3). TMA was variable between hedgerows in both years of harvest, with relative standard deviation (RSD) values between 16% and 30%, yet there was not a significant difference in the overall average TMA between 2018 and 2019 (Table 3). Furthermore, most hedgerows were not significantly different from the other hedgerows harvested that year (Table 3) despite significant differences in TMA values found between farms in both years (Table S2). Regarding the age of the elderberry shrub, hedgerows 2 and 14 (bare root, pre-rooted cuttings planted in 2018) had two of the three highest concentrations of TMA in 2019 (111.5 ± 9.4 and 109.2 ± 12.6 mg CGE per 100 g FW, respectively). This suggests that blue elderberries can be harvested from plants as young as two years without a significant loss of TMA concentrations.

TMA values for the blue elderberries are lower than those found in other elderberry subspecies. In European elderberries, TMA levels range from 170 ± 12 to 343 ± 11 with an average of 239 ± 94 mg CGE per 100 g FW [49]. A study of American elderberry grown in Ohio showed a range from 354 ± 59 to 595 ± 26 mg CGE per 100 g FW.[52] In the present study, bare root pre-rooted cuttings of American elderberries were planted, along with blue elderberries, on Farm 1 in 2018, and three shrubs were harvested in 2019. These American elderberries had an average TMA value of 263 ± 5.4 mg CGE per 100 g FW, which is more similar to what has been observed in other studies on this subspecies. This suggests it is a subspecies difference contributing to the lower anthocyanin concentration in the blue elderberry and not the difference in growing conditions. Compared to other berries, blue elderberries have similar levels of anthocyanins as raspberries, but lower levels than blueberries and blackberries [100]. The lower concentration of anthocyanins in the

blue elderberry may require adjustment of levels used in supplements, food and beverages for optimal performance or health benefit, or as natural coloring agents.

Table 3. Total monomeric anthocyanin (TMA) and total phenolic content (TPC) data from each blue elderberry hedgerow harvested in 2018 and 2019

hedgerow	TMA [a]				TPC [b]			
	n (2018)	2018	n (2019)	2019	n (2018)	2018	n (2019)	2019
1	7	100.0 ± 9.7 b	9	80.5 ± 9.4 abc	7	714 ± 31 a	9	640 ± 34 ab
2	0	not harvested	10	111.5 ± 9.4 bc	0	not harvested	10	554 ± 34 ab
3	5	64.1 ± 11.5 ab	4	68.3 ± 12.6 abc	5	538 ± 37 ab	4	459 ± 59 a
4	6	89.3 ± 10.5 b	6	78.5 ± 11.5 abc	7	544 ± 31 ab	6	483 ± 41 a
5	7	34.2 ± 9.7 a	5	43.1 ± 11.5 a	7	529 ± 31 a	6	533 ± 45 ab
6	6	64.6 ± 10.5 ab	6	60.9 ± 12.6 ab	6	604 ± 34 abc	6	493 ± 41 ab
7	6	104.8 ± 10.5 b	6	121.5 ± 11.5 c	6	791 ± 34 d	6	695 ± 41 b
8	5	65.8 ± 11.5 ab	4	68.9 ± 14.1 abc	5	705 ± 37 bcd	4	636 ± 50 ab
9	2	113.4 ± 18.2 b	2	93.5 ± 20.0 abc	2	692 ± 58 abcd	2	584 ± 71 ab
10	5	62.3 ± 11.5 ab	5	64.1 ± 12.6 abc	5	610 ± 37 abc	5	519 ± 45 ab
11	2	73.8 ± 18.2 ab	1	59.7 ± 28.3 abc	2	732 ± 58 abcd	1	685 ± 101 ab
12	5	82.9 ± 11.5 ab	6	89.8 ± 11.5 abc	5	634 ± 37 abcd	6	502 ± 41 ab
13	6	68.3 ± 10.5 ab	6	97.8 ± 11.5 abc	4	514 ± 41a	6	525 ± 41ab
14	0	not harvested	6	109.2 ± 12.6 bc	0	not harvested	6	574 ± 41ab
Avg	62	76.8 ± 22.2 a	68	79.6 ± 12.9 a	62	637 ± 97 a	76	561 ± 76 b

[a] mg CGE per 100 g FW. [b] mg GAE per 100 g FW. Average values ± standard error are presented. Different letters (a-d) in columns, excluding the average, indicate statistically significant differences between hedgerows ($p < 0.05$) according to Tukey's test. Difference letters for average concentrations indicate significant differences between the harvest years.

3.3 Total phenolic content

In addition to anthocyanins, elderberries contain other phenolic compounds, such as flavonols and phenolic acids [49], which also contribute to the health promoting properties of elderberry [26]. Phenolic compounds are responsible for organoleptic properties (e.g., flavor, color, and mouthfeel) and can help protect foods against lipid oxidation. Therefore, TPC can be useful for making approximate comparisons, for example, between varieties of the same fruit, between similar fruits or in the evaluation of a processing step (i.e., general increases or decreases). It is important to note that the TPC assay is a non-selective assay and is easily impacted by extraction conditions and interfering substances, such as ascorbic acid and reducing sugars. Although there is no evidence that the beneficial effects of polyphenol-rich foods can be attributed to the TPC of a food, it can be a useful measure for making general comparisons with other studies in the literature which reported these values but should be supported by quantitative HPLC data.

Herein, the range of TPC measured in the blue elderberries was from 514 ± 41 to 791 ± 34 mg GAE per 100 g FW in 2018 and from 459 ± 50 to 695 ± 41 mg GAE per 100 g FW in 2019 (Table 3). TPC in the blue elderberries was significantly higher ($p < 0.05$) in 2018 (637 ± 97 mg GAE per 100 g FW) than in 2019 (561 ± 76 mg GAE per 100 g FW) (Table 3). While there were significant differences found between the farms in both years (Table S2), most hedgerows were not significantly different than most other hedgerows in the given year when evaluated together (Table 3). Although the farms in this study were near each other and experience similar climates, there can still be differences in growing conditions for each hedgerow, such as water availability, which has been shown to influence the levels of phenolics in blueberries [101] and strawberries [102]. Hedgerows 2 and 14 (bare root, pre-rooted cuttings planted in 2018) were not significantly different from other hedgerows in 2019, indicating that the blue elderberries can be harvested early

in the plant's lifetime, which allows farmers to earn an early return on the investment of establishing hedgerows. The TPC in blue elderberry is similar to those found in other elderberry species [18,29,49,50,52,95,103]. These comparisons show that blue elderberries from hedgerows are a rich source of phenolic compounds.

3.4 Quantitation of phenolic compounds

Phenolic compounds were identified and quantified in the blue elderberry based upon retention time, absorbance spectra and authentic standards when available. Concentrations for samples from 2018 are presented in Table 4, while samples from 2019 are presented in Table 5. Two peaks with significant area were observed in the HPLC chromatograms at 6.96 min and 11.70 min that did not correlate to standards or library matching. Both compounds eluted between the retention time of gallic acid and protocatechuic acid. The first eluting compound had a maximum absorbance at 300 nm while the second compound had a maximum absorbance at 280 nm. These peaks were collected individually and further evaluated by accurate mass quadrupole time-of-flight tandem mass spectrometry (QTOF-MS/MS). TOF acquires mass spectral data by pulsing ions entering the flight tube in an orthogonal beam, therefore full spectra are collected. The data captured is accurate enough to determine the elemental composition therefore allowing identification without standards [104]. The two compounds were tentatively identified using high mass accuracy (within 1 part per million) as 5-hydroxypyrogallol (5-HPG) hexoside, a tetrahydroxybenzene (Figure 1), and protocatechuic acid (PA) dihexoside (Figure 2). Accurate mass was especially helpful since commercial standards for these compounds are not available. 5-HPG hexoside was identified by its fragmentation pattern (Figure 1), showing a precursor ion $[M-H]^-$ at *m/z* 303.0723 and product ion $[M\text{-hexose-H}]^-$ at *m/z* 141.0186 (Figure 3a). This compound was one of the most abundant phenolic compounds in the blue elderberry. While no evidence of

5-HPG glycoside was found in the literature, the aglycone has shown to have a high radical scavenging activity compared to other simple phenols [105].

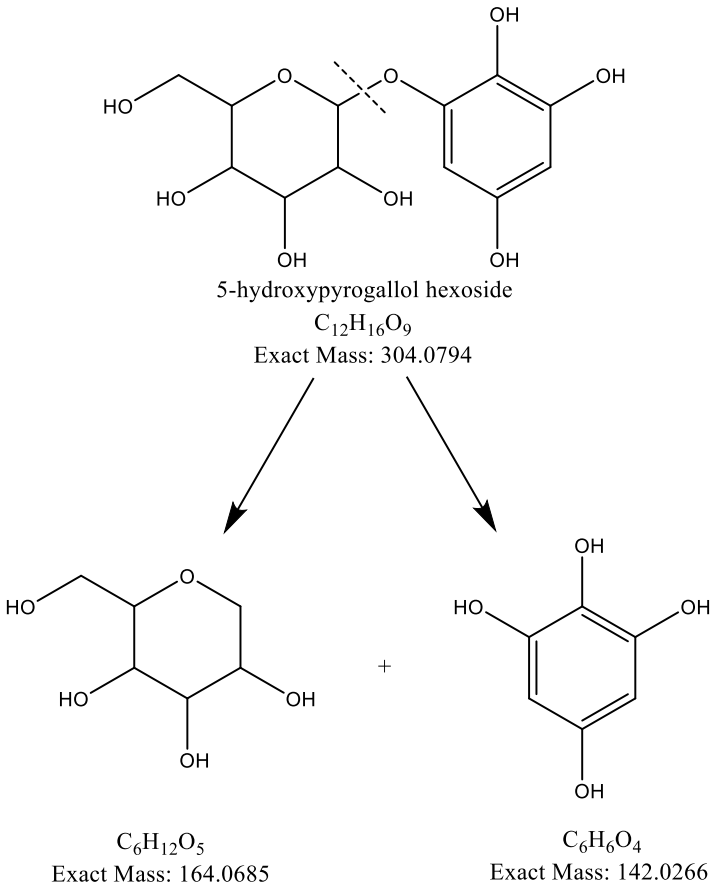


Figure 1. Fragmentation of 5-hydroxypyrogallol hexoside

The other novel phenolic compound identified was protocatechuic acid (PA) dihexoside, also present in relatively high amount in almost all the samples. The precursor ion $[M-H]^-$ at *m/z* 477.1609 fragmented to give product ions corresponding to [dihexoside – H]$^-$ at m/z 323.0981 and [M-dihexose -H]$^-$ at *m/z* 153.0562 m/z (Figure 3b). The loss of 324 amu has been identified as the loss of a dihexoside on other phenolic compounds and was proposed to be sophorose or gentiobiose

[106]. PA is a breakdown product of cyanidin-based anthocyanins and has been quantified in elderberry juice during thermal processing [107]. PA has been shown to have pharmacological potential in the prevention and/or treatment of neurodegenerative diseases in humans based on *in vitro* and i*n vivo* studies [108].

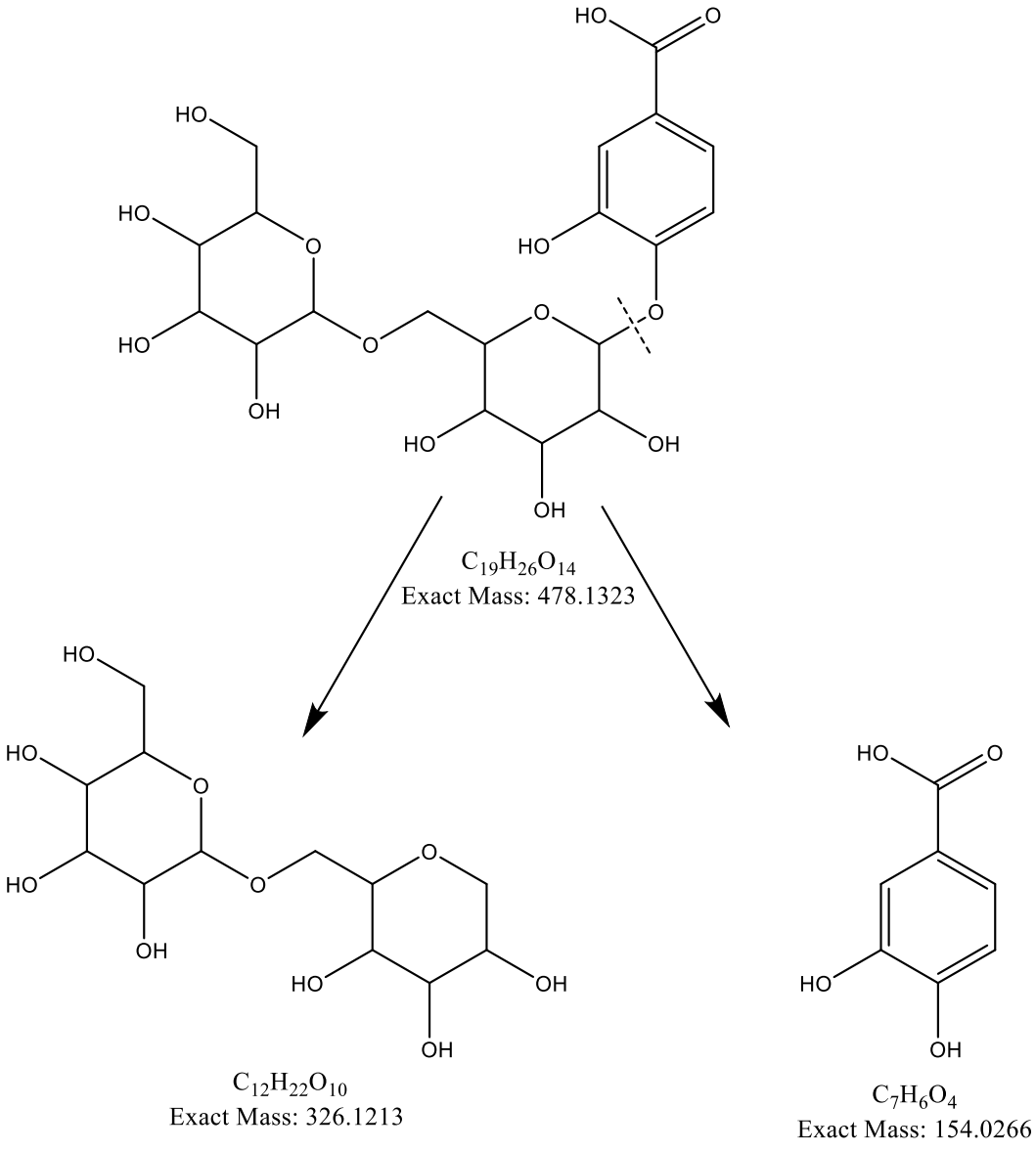
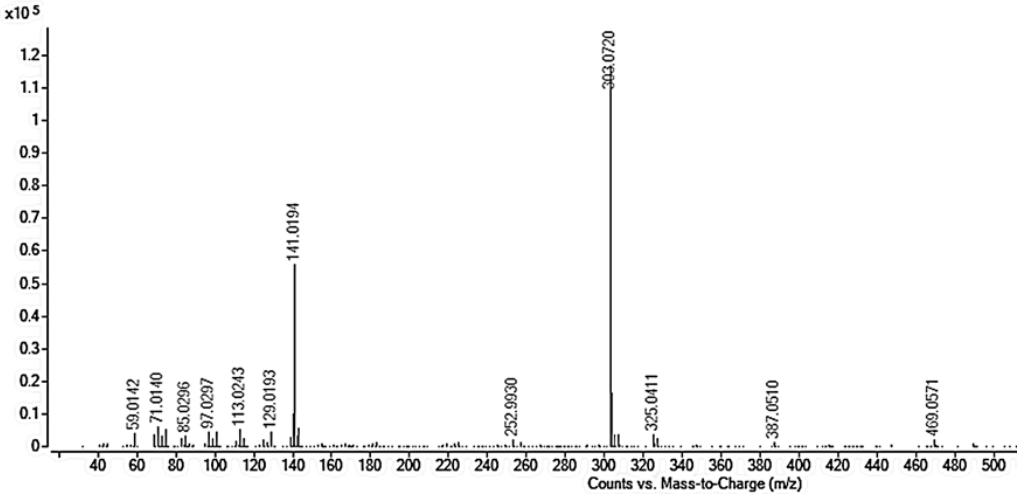


Figure 2. Fragmentation of protocatechuic acid dihexoside

a)



b)

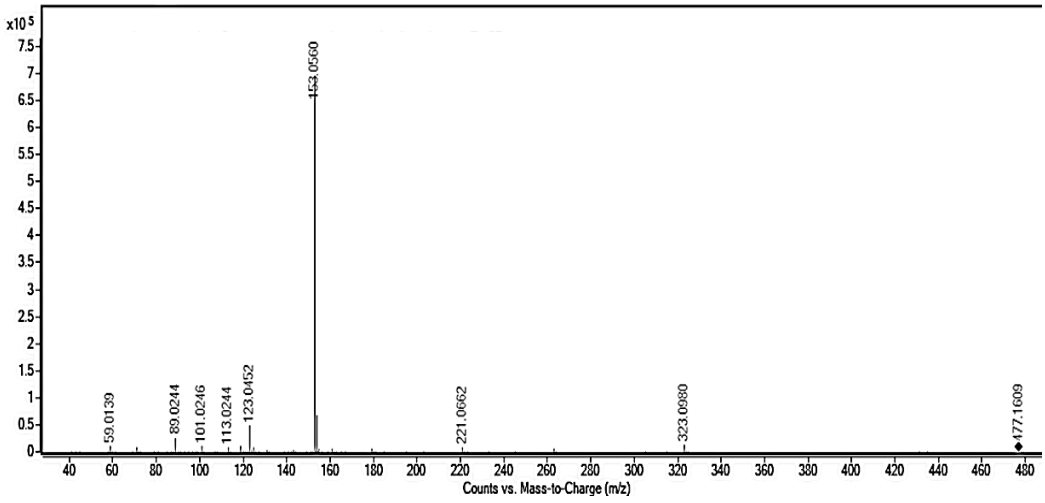


Figure 3. Negative QTOF-MS/MS fragmentation spectra of a) 5-hydroxypyrogallol hexoside, and b) protocatechuic acid di-hexoside.

Other phenolic acids identified in the berries include neochlorogenic acid and chlorogenic acid, which is the major isomer present in the other subspecies. The levels of chlorogenic in blue elderberry, which averaged 16.68 ± 2.25 mg per 100 g FW in 2018 and 14.24 ± 3.37 mg per 100 g FW in 2019, are similar to American elderberry (average of 14.1 ± 4.5 mg per 100 g FW) [49] and

European elderberry in one study (15.38 ± 1.89 mg per 100 g FW) [59]. However the levels in the present study are about half of the levels found in European elderberry in another study (31.3 ± 4.7 mg per 100 g FW) [49] and they are also lower than that found in European-grown *S. nigra* ssp. *cerulea*, which averaged 24.95 ± 1.26 mg per 100 g FW [59]

Table 4. Concentration of phenolic compounds present in elderberries harvested in 2018 (average ± standard deviation, n=3) in mg per100 g FW

hedgerow	1	3	4	5	6	7
5-HPG hexoside	12.79 ± 9.84 abc	nd	56.97 ± 10.59 e	9.31 ± 6.72 ab	37.97 ± 10.67 cde	10.69 ± 3.65 ab
PA dihexoside	42.84 ± 4.54 bc	49.12 ± 8.46 c	30.30 ± 5.59 abc	31.84 ± 11.86 abc	47.49 ± 5.73 c	45.06 ± 1.29 bc
Neochlorogenic acid	5.29 ± 0.41 de	3.89 ± 1.25 bcde	6.19 ± 1.10 e	2.64 ± 0.68 abc	2.94 ± 0.62 abcd	4.90 ± 1.39 cde
Chlorogenic acid	16.22 ± 3.04 a	20.47 ± 1.25 a	15.31 ± 1.45 a	17.00 ± 2.70 a	18.11 ± 9.01 a	17.72 ± 2.34 a
Rutin	59.18 ± 8.11 abc	36.43 ± 19.14 ab	87.52 ± 3.24 d	35.49 ± 4.98 a	51.05 ± 10.85 abc	52.53 ± 4.80 abc
Isoquercetin	5.24 ± 0.75 a	2.54 ± 0.45 a	4.56 ± 0.96 a	1.94 ± 0.10 a	2.48 ± 0.36 a	4.00 ± 0.18 a
Kaempferol-3-rutinoside	5.09 ± 1.14 abcd	3.75 ± 1.72 abc	7.73 ± 0.73 d	3.00 ± 0.48 a	3.23 ± 0.70 ab	6.06 ± 1.33 bcd
Isorhamnetin-3-rutinoside	44.79 ± 31.99 ab	21.50 ± 21.03 ab	39.35 ± 6.16 ab	15.01 ± 7.67 a	45.95 ± 24.36 ab	42.02 ± 8.15 ab
Cyanidin-3-sambubioside-5-glucoside	4.88 ± 0.72 bcde	5.78 ± 0.93 bcde	6.53 ± 2.65 cde	3.93 ± 0.20 abcd	4.38 ± 1.59 abcde	1.31 ± 1.37 a
Cyanidin-3,5-diglucoside	26.77 ± 2.30 a	17.11 ± 11.91 a	22.80 ± 1.35 a	11.92 ± 0.23 a	16.17 ± 5.67 a	17.67 ± 1.06 a
Cyanidin-3-sambubioside	40.53 ± 4.88 bcde	23.58 ± 8.26 bcde	48.29 ± 5.74 cde	20.07 ± 2.38 abcd	18.18 ± 3.65 abcde	35.62 ± 9.16 a
Cyanidin-3-glucoside	4.51 ± 2.66 a	9.28 ± 8.98 a	4.32 ± 1.38 a	1.85 ± 0.22 a	2.44 ± 0.29 a	7.07 ± 6.61 a
Catechin	3.44 ± 1.40 ab	2.82 ± 0.69 ab	2.72 ± 0.32 ab	4.11 ± 0.39 abc	2.80 ± 1.24 ab	7.51 ± 1.75 d
Epicatechin	8.38 ± 0.93 bc	3.85 ± 0.92 a	5.33 ± 1.75 ab	7.44 ± 0.27 abc	3.87 ± 0.48 a	8.14 ± 1.58 bc

Values within a row that do not share a letter are significantly different according to Tukey's HSD ($p < 0.05$). nd stands for not detected.

Table 4. continued

hedgerow	8	9	10	11	12	13	Overall average
5-HPG hexoside	31.46 ± 20.93 bcde	2.74 ± 0.33 a	19.13 ± 8.04 abcd	4.52 ± 0.32 a	6.68 ± 6.61 ab	44.30 ± 5.37 de	21.50 ± 18.32
PA dihexoside	20.98 ± 2.71 a	44.85 ± 13.08 bc	42.08 ± 4.62 bc	14.39 ± 0.51 a	21.23 ± 1.90 a	26.12 ± 3.59 ab	34.69 ± 12.01
Neochlorogenic acid	1.78 ± 0.22 ab	4.92 ± 0.60 cde	2.00 ± 0.53 ab	0.41 ± 0.02 a	5.13 ± 1.33 cde	5.12 ± 1.17 cde	3.77 ± 1.78
Chlorogenic acid	13.38 ± 6.98 a	17.49 ± 3.30 a	12.83 ± 1.39 a	19.13 ± 0.24 a	17.52 ± 2.67 a	14.94 ± 1.70 a	16.68 ± 2.25
Rutin	87.12 ± 6.63 d	55.99 ± 9.17 abc	38.55 ± 32.97 abc	62.83 ± 4.45 bcd	68.01 ± 11.89 cd	49.40 ± 6.87 abc	57.01 ± 17.42
Isoquercetin	17.23 ± 24.58 a	2.51 ± 0.71 a	4.86 ± 0.27 a	4.82 ± 0.31 a	2.82 ± 0.38 a	2.80 ± 0.24 a	4.52 ± 4.14
Kaempferol-3-rutinoside	11.36 ± 13.16 cd	4.29 ± 0.16 abc	3.96 ± 2.57 abcd	46.47 ± 35.56 abcd	3.91 ± 0.44 abc	4.15 ± 1.12 abc	8.58 ± 12.16
Isorhamnetin-3-rutinoside	15.76 ± 12.11 a	21.60 ± 3.07 ab	41.35 ± 0.80 ab	64.57 ± 4.39 b	24.35 ± 10.06 ab	20.60 ± 0.24 a	28.30 ± 14.03
Cyanidin-3-sambubioside-5-glucoside	2.70 ± 0.49 ab	6.62 ± 1.09 de	2.87 ± 0.70 abc	10.19 ± 0.48 e	6.80 ± 0.36 de	4.15 ± 1.12 de	5.01 ± 2.36
Cyanidin-3,5-diglucoside	14.81 ± 3.21 a	21.15 ± 8.29 a	16.01 ± 5.97 a	31.80 ± 0.72 a	24.35 ± 1.50 a	20.57 ± 12.97 a	20.11 ± 5.63
Cyanidin-3-sambubioside	26.86 ± 5.32 ab	37.20 ± 5.66 de	20.87 ± 8.19 abc	40.69 ± 25.00 e	41.15 ± 4.13 de	39.30 ± 4.30 de	32.70 ± 10.68
Cyanidin-3-glucoside	3.02 ± 1.56 a	2.55 ± 0.31 a	1.53 ± 0.33 a	5.03 ± 0.17 a	2.94 ± 0.58 a	2.86 ± 0.94 a	3.72 ± 2.30
Catechin	4.79 ± 1.19 bcd	2.90 ± 0.41 ab	3.30 ± 1.32 ab	5.20 ± 0.15 cd	1.96 ± 0.51 a	2.19 ± 0.50 a	3.65 ± 1.56
Epicatechin	8.57 ± 1.51 c	6.66 ± 1.90 abc	6.99 ± 1.84 abc	13.92 ± 0.01 a	6.95 ± 1.29 abc	1.90 ± 1.28 ab	6.83 ± 3.05

Values within a row that do not share a letter are significantly different according to Tukey's HSD ($p < 0.05$). nd stands for not detected.

Table 5. Concentration of phenolic compounds present in elderberries harvested in 2019 (average ± standard deviation, n=3) in mg/100 g FW

hedgerow	1	2	3	4	5	6	7
5-HPG hexoside	12.63 ± 5.15 a	93.22 ± 8.80 d	20.30 ± 2.25 ab	36.67 ± 6.10 b	2.27 ± 1.69 a	21.46 ± 6.80 ab	4.26 ± 6.52 a
PA dihexoside	49.64 ± 10.85 fg	19.44 ± 0.78 a	27.48 ± 2.43 abcd	24.76 ± 1.14 abc	45.86 ± 9.01 efg	39.13 ± 4.89 bcdef	41.09 ± 6.59 cdefg
Neochlorogenic acid	5.28 ± 1.27 cde	5.78 ± 1.45 de	3.34 ± 0.47 abcd	4.01 ± 0.93 abcd	2.26 ± 0.04 ab	1.57 ± 0.25 a	5.44 ± 0.61 de
Chlorogenic acid	16.59 ± 1.52 ab	11.46 ± 3.42 a	19.15 ± 0.23 b	10.01 ± 1.03 a	14.50 ± 1.22 ab	19.70 ± 6.03 b	19.05 ± 1.25 b
Rutin	51.78 ± 1.37 ab	101.07 ± 10.49 c	32.44 ± 1.32 ab	62.64 ± 6.73 b	26.95 ± 4.51 a	48.69 ± 10.79 ab	56.09 ± 4.50 ab
Isoquercetin	3.76 ± 0.33 cd	3.74 ± 0.88 cd	1.64 ± 0.07 a	20.24 ± 2.15 abcd	1.76 ± 0.39 ab	2.81 ± 0.25 abcd	2.78 ± 0.08 abcd
Kaempferol-3-rutinoside	5.33 ± 1.07 bcd	6.72 ± 0.96 d	2.56 ± 0.11 a	62.64 ± 6.73 abc	2.77 ± 0.28 a	20.12 ± 16.27 abc	3.71 ± 0.13 abc
Isorhamnetin-3-rutinoside	49.77 ± 11.19 d	49.12 ± 10.61 d	23.17 ± 0.80 abc	26.96 ± 6.48 abcd	10.96 ± 3.33 ab	35.51 ± 14.77 bcd	38.47 ± 8.31 cd
Cyanidin-3-sambubioside-5-glucoside	3.70 ± 0.44 ab	5.81 ± 0.86 ab	4.26 ± 0.29 ab	1.98 ± 0.29 a	3.61 ± 0.70 ab	11.15 ± 0.90 ab	10.67 ± 9.05 b
Cyanidin-3,5-diglucoside	22.92 ± 4.34 cd	22.58 ± 2.78 cd	17.07 ± 1.02 abc	9.69 ± 2.19 c	9.02 ± 1.04 a	18.61 ± 0.90 abc	30.43 ± 6.53 de
Cynanidin-3-sambubioside	34.86 ± 7.17 ab	55.53 ± 8.80 ab	21.05 ± 1.55 ab	2.50 ± 1.05 a	16.53 ± 1.64 ab	7.36 ± 0.91 ab	33.00 ± 16.78 b
Cyanidin-3-glucoside	3.35 ± 0.38 a	3.61 ± 0.14 a	2.84 ± 0.24 a	3.13 ± 0.53 a	1.75 ± 0.09 a	1.68 ± 0.35 a	5.74 ± 5.50 a
Catechin	3.17 ± 0.08 abc	2.42 ± 0.41 a	1.80 ± 0.12 a	2.37 ± 1.80 a	2.39 ± 0.58 a	2.91 ± 0.65 ab	5.06 ± 0.58 bcd
Epicatechin	6.07 ± 1.00 a	4.54 ± 1.19 a	3.38 ± 0.20 a	2.60 ± 1.79 a	5.37 ± 0.97 a	3.48 ± 0.20 a	4.93 ± 1.60 a

Values within a row that do not share a letter are significantly different according to Tukey's HSD ($p < 0.05$). nd stands for not detected.

Table 5. continued

hedgerow	8	9	10	11	12	13	14	Overall average
5-HPG hexoside	21.21 ± 6.98 ab	nd	13.84 ± 5.54 a	3.83 ± 0.20 a	20.79 ± 9.70 ab	40.72 ± 18.06 bc	60.88 ± 5.93 c	27.08 ± 25.83
PA dihexoside	31.36 ± 0.66 abcde	57.00 ± 3.62 g	42.15 ± 3.67 defg	22.98 ± 0.56 ab	45.11 ± 4.53 efg	34.21 ± 11.19 abcdef	18.32 ± 4.44 a	35.61 ± 11.96
Neochlorogenic acid	4.39 ± 0.20 bcd	2.79 ± 0.47 abc	3.40 ± 0.29 abcd	2.18 ± 0.29 ab	5.03 ± 0.08 cde	5.71 ± 1.25 de	7.17 ± 1.65 e	4.17 ± 1.64
Chlorogenic acid	16.37 ± 0.89 ab	12.07 ± 0.58 a	12.06 ± 0.52 a	13.05 ± 0.31 ab	13.27 ± 3.10 ab	12.18 ± 1.22 a	9.91 ± 2.11 a	14.24 ± 3.37
Rutin	60.20 ± 6.69 b	50.14 ± 3.93 ab	50.37 ± 3.57 ab	36.89 ± 3.87 ab	51.93 ± 12.05 ab	57.11 ± 11.57 ab	103.40 ± 32.38 c	51.89 ± 25.53
Isoquercetin	3.50 ± 0.45 bcd	3.09 ± 0.26 abcd	3.88 ± 0.35 d	3.93 ± 0.55 d	2.04 ± 0.57 abcd	3.15 ± 1.23 abc	3.09 ± 0.30 abcd	4.24 ± 4.67
Kaempferol-3- rutinoside	4.32 ± 1.18 abcd	4.21 ± 0.52 abc	24.35 ± 17.04 abcd	4.41 ± 0.81 abcd	3.42 ± 1.14 ab	4.49 ± 1.25 abcd	6.02 ± 1.09 cd	11.08 ± 16.24
Isorhamnetin-3-rutinoside	6.91 ± 7.42 a	16.10 ± 1.07 abc	32.72 ± 4.14 bcd	32.54 ± 4.91 abcd	14.44 ± 8.27 abc	29.13 ± 13.35 abcd	36.91 ± 10.86 cd	24.71 ± 14.83
Cyanidin-3-sambubioside-5-glucoside	2.81 ± 0.26 a	4.61 ± 0.45 ab	12.00 ± 0.66 ab	6.80 ± 0.27 ab	7.30 ± 0.63 ab	6.93 ± 1.62 ab	5.51 ± 1.08 ab	6.22 ± 3.15
Cyanidin-3,5-diglucoside	18.66 ± 1.99 bc	23.55 ± 3.93 cde	20.45 ± 2.07 abc	10.14 ± 0.28 ab	19.06 ± 0.66 c	31.77 ± 3.31 e	23.25 ± 2.68 cde	19.80 ± 6.92
Cynanidin-3-sambubioside	33.57 ± 5.84 a	35.61 ± 3.95 ab	7.97 ± 3.27 ab	30.50 ± 1.54 ab	39.83 ± 5.05 ab	39.68 ± 6.93 ab	57.24 ± 8.86 ab	29.66 ± 16.81
Cyanidin-3-glucoside	5.62 ± 0.40 a	1.51 ± 0.08 a	2.10 ± 0.13 a	2.53 ± 0.21 a	2.31 ± 0.24 a	4.12 ± 0.34 a	3.85 ± 1.09 a	3.15 ± 1.35
Catechin	6.19 ± 0.76 d	2.03 ± 0.14 a	4.50 ± 0.74 abc	5.57 ± 0.47 cd	1.26 ± 0.26 a	2.60 ± 1.77 a	1.49 ± 0.24 a	3.12 ± 1.57
Epicatechin	10.59 ± 1.13 b	5.47 ± 0.14 a	4.29 ± 0.82 a	3.37 ± 0.14 a	3.79 ± 0.64 a	4.78 ± 1.95 a	3.50 ± 1.53 a	4.73 ± 1.95

Values within a row that do not share a letter are significantly different according to Tukey's HSD ($p < 0.05$). nd stands for not detected.

Like other elderberry species, rutin (quercetin-3-rutinoside) was the predominant flavonol and overall had the highest concentration of any of the flavonols measured, with an average of 57.01 ± 17.42 mg per 100 g FW in 2018 and 51.89 ± 25.53 mg per 100 g FW in 2019. These values fall within the range of what has been found in European elderberry [8,42,49,59]. Other flavonols identified include isoquercetin (quercetin-3-glucoside), kaempferol-3-rutinoside, and isorhamnetin-3-rutinoside, which was also a major phenolic compound in the berry. Isorhamnetin-3-rutinoside averaged 28.30 ± 14.03 mg per 100 g FW in 2018 and 24.71 ± 14.83 mg per 100 g FW in 2019, which is higher than what has been found in other subspecies[42]. Overall, the blue elderberry analyzed in the present study has much higher levels of total flavonols as compared to European elderberry [6,59]. In the American elderberry, the main flavonols are rutin followed by isorhamnetin-3-rutinoside whereas in European elderberries, the main flavonols are rutin followed by isoquercetin [49,59]. In blue elderberry grown in Slovenia, rutin and isoquercetin were the two predominant flavonols, though the total flavonols in found for the subspecies (81.6 ± 3.52 mg per 100 g FW) was similar to the levels found in this study [59].

The predominant anthocyanin present in the blue elderberry is cyanidin-3-sambubioside, like the European subspecies. The average concentration in 2018 was 32.70 ± 10.18 mg per 100 g FW and 29.66 ± 16.81 mg per 100 g FW in 2019. Cyanidin-3,5-diglucoside was the next most concentrated anthocyanin, averaging 20.11 ± 5.63 mg per 100 g FW in 2018 and 19.80 ± 6.92 mg per 100 g FW in 2019. This is unlike European elderberries, in which cyanidin-3-glucoside is typically the second most prominent anthocyanin, except for the Ljubostinja cultivar which has more cyanidin-3,5-diglucoside than cyanidin-glucoside[42]. Cyanidin-3-sambubioside-5-glucoside and cyanidin-3-glucoside were also quantified in the berries. Cyanidin-3,5-diglucoside and cyanidin-3-sambubioside-5-glucoside were not detected in blue elderberries grown in Slovenia,

suggesting the growing location impacts the profile of phenolic compounds or perhaps the two samples going by the same name are not, in fact, related [109]. There were no acylated anthocyanins identified in the blue elderberry, like those abundant in the American elderberry. Overall, total anthocyanin concentrations averaged 61.54 ± 16.70 mg per 100 g FW in 2018 and 58.58 ± 22.18 mg per 100 g FW in 2019. The total concentration of anthocyanins in the berries was much lower compared to the other subspecies [8,49,109]. Analysis of European elderberries that measured cyanidin-based anthocyanins found an average of 863.8 ± 49.9 mg per 100 g FW [8]. European elderberries grown in different locations at different altitudes had a range of 289.74 ± 66.18 to 792.66 ± 27.97 mg per 100 g FW [6]. In studies on American elderberries, one had an average of 265 ± 74 mg per 100 g FW [49], another had average of 248 ± 83 mg per 100 g FW [18], and a third had an average of 242.7 ± 91.0 mg per 100 g FW [50].

The flavan-3-ols catechin and epicatechin were measured in the elderberry, with epicatechin typically present in higher concentrations. The concentrations found in the present study are similar to those found in others, even across subspecies. Blue elderberry grown in Slovenia had 4.40 ± 0.26 mg per 100 g FW of catechin and 8.49 ± 0.37 mg per 100 g FW of epicatechin. The same study found no catechin present in *S. nigra* ssp. *nigra*, but 6.37 ± 0.28 mg per 100 g FW of epicatechin [59]. In a study of European berries growing in different locations at different altitudes, total flavanol concentrations ranged from 1.93 ± 0.22 to 9.67 ± 0.66 mg per 100 g FW [6].

The variability in phenolic and anthocyanin content observed in this study is not surprising, as multiple other studies have shown significant variability in other commercialized elderberry subspecies, even with clonally propagated cultivars. For example, Lee and Finn (2007)[49] saw an average of 45% higher anthocyanins in their second harvest of American elderberries grown in

Oregon as compared to their first harvest, though the total phenolics only increased an average of 20%. Johnson et al. (2017)[54] observed significant changes between subsequent years in anthocyanin and phenolic compound concentrations in juices prepared from American elderberry grown in two locations in Missouri. For example, in the Adams II sample grown in one Missouri location, the quercetin 3-rutinoside content was 298 ± 48 mg L^{-1} in 2012, 792 ± 143 mg L^{-1} in 2013, and 47 ± 13 mg L^{-1} in 2014 [54]. In a study of 107 wild American elderberries samples grown in five regions of the eastern United States by Mudge et al. (2016)[110] high variability was found in selected flavonoid compounds (chlorogenic acid, rutin, quercetin, and isoquercetin) with an average RSD of 55.3% across samples. Overall, there is a body of evidence demonstrating that elderberry composition can vary year to year or by growing conditions even in clonally-propagated cultivars; therefore, it may be necessary to use standardization techniques for bioactive compounds in order to maintain consistent quality in elderberry products.

Blue elderberry (*S. nigra* ssp. *cerulea*) grown in California hedgerows has similar levels of sugar, organic acids, and TPC to the European (*S. nigra* ssp. *nigra*) and American (*S. nigra* ssp. *canadensis*) elderberry subspecies. Furthermore, the phenolic profile of blue elderberry is similar to European elderberry, in that chlorogenic acid, rutin, and cyanidin-3-sambubioside are the predominant hydroxycinnamic acid, flavonol, and anthocyanin, respectively. However, anthocyanin levels are significantly lower in the blue elderberry compared to European and American subspecies, yet the levels of total flavonols appears to be much higher than the other subspecies. 5-Hydroxypyrogallol hexoside and protocatechuic acid dihexoside were identified for the first time in elderberry, which could potentially serve as markers of this subspecies in products that use blue elderberry. There was considerable variation within and between hedgerows in both harvest years, but this appears to be a common attribute for the elderberry species. Blue

elderberries have many ecological benefits for farms when planted in hedgerows, grow well in challenging environments, are not killed by wildfires and can therefore, serve as a sustainable source of an increasingly popular fruit.

Abbreviations used

5-HPG: 5-hydroxypyrogallol; ANOVA: analysis of variance; ACN: acetonitrile; CGE: cyanidin-glucoside equivalents; DAD: diode array detector; FLD: fluorescence detector; FW: fresh weigh

GAE: gallic acid equivalents; HPLC: high performance liquid chromatography; LLOD: lower limit of detection; LLOQ: lower limit of quantitation; MeOH: methanol; MS: mass spectrometry; NaOH: sodium hydroxide; PA: protocatechuic acid; QTOF: quadrupole time of flight; RSD: relative standard deviation; SE: standard error; TA: titratable acidity; TMA: total monomeric anthocyanins; TPC: total phenolic content

Author contributions

KU: methodology, conducting analyses, writing and editing the draft of the manuscript. KF: project conceptualization, funding and sample acquisition, reviewing draft of manuscript. SB: project conceptualization, funding acquisition and administration, reviewing draft of manuscript. AM: project conceptualization, funding administration, experimental design, supervision, editing and reviewing draft of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Table S1. Calibration curve parameters, including linear equation, correlation coefficient, lower limit of detection (LLOD) and lower limit of quantification (LLOQ) in parts per million (ppm) averaged over four replicates

	Linear equation	R2	LLOD (ppm)	LLOD (ppm)
Chlorogenic acid	y = 13.762x – 12.901	0.9998	1.09	3.29
Rutin	y = 6.5514x – 6.0649	0.9998	0.97	2.93
Cyanidin-3-glucoside	y = 19.811x – 30.259	0.9996	0.46	1.39
Catechin	y = 52.568x – 1.1162	0.9992	0.48	1.38

Table S2. Total monomeric anthocyanin (TMA) and total phenolic content (TPC) data from each farm harvested in 2018 and 2019

farm	TMA [a]		TPC [b]	
	2018	2019	2018	2019
1	100.0 ± 9.7 b	96.0 ± 6.7 b	714 ± 31 b	597 ± 24 b
2	62.5 ± 6.1 a	63.3 ± 6.9 a	537 ± 19 a	492 ± 26 a
3	82.2 ± 5.7 ab	81.8 ± 6.5 ab	680 ± 18 b	585 ± 23 ab
4	75.0 ± 8.0 ab	82.4 ± 10.9 ab	627 ± 27 b	570 ± 39 ab
5	not harvested	109.2 ± 12.6 b	not harvested	574 ± 41 ab

[a] mg CGE per 100 g FW. [b] mg GAE per 100 g FW. Average values ± standard error are presented. Different letters (a-d) in columns indicate statistically significant differences between farms ($p < 0.05$) according to Tukey's test.

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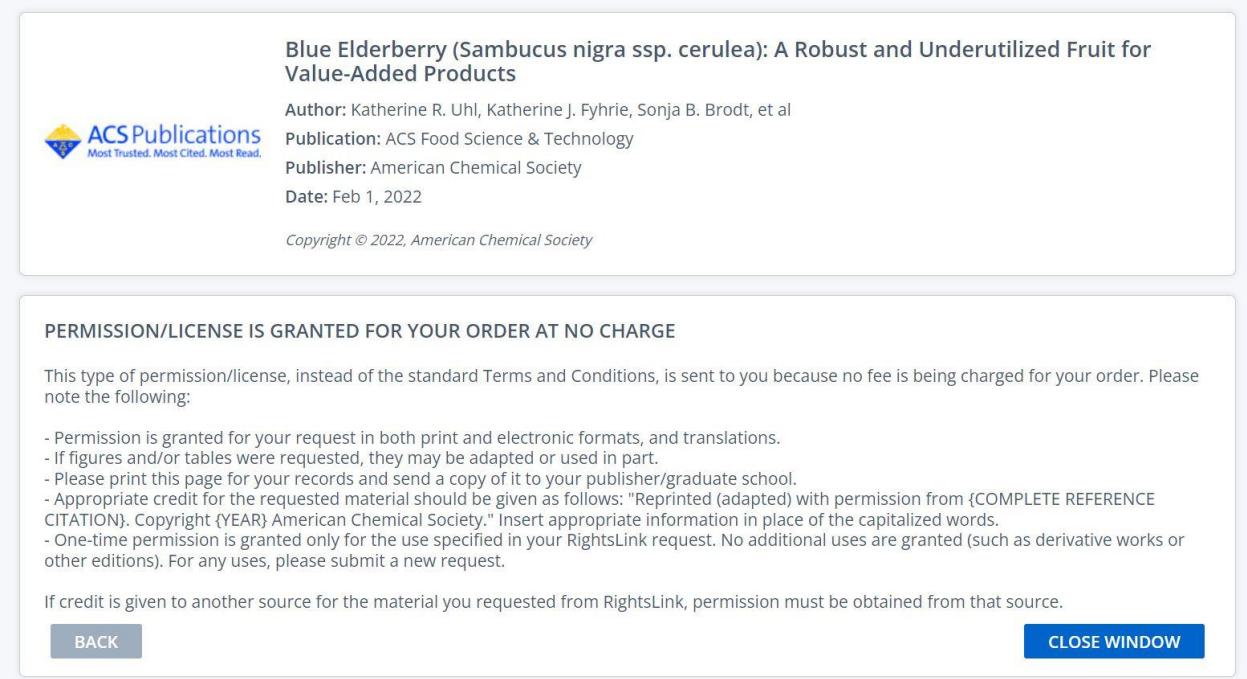

Blue Elderberry (Sambucus nigra ssp. cerulea): A Robust and Underutilized Fruit for Value-Added Products

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Chapter 2. Headspace Volatile Organic and Phenolic Compounds in Elderflowers and Elderflower Teas of the Blue Elderberry (*Sambucus nigra* ssp. *cerulea*)

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Abstract

Elderflower products have become more popular in the U.S., yet most are still made from European-grown flowers. Flowers of the subspecies native to the western region of North America (*Sambucus nigra* ssp. *cerulea*) were investigated for the first time. The phenolic compounds were evaluated in ethanol/water extracts of fresh and dry flowers (either homogenized or as the intact flower), and in hot water extracts (teas) of the dry, intact flowers. Fresh homogenized flowers had significantly higher levels of phenolic compounds than the other preparations. The predominant flavonols identified were isorhamnetin-3-*O*-rutinoside (ranging from 32.48 to 78.73 mg g^{-1} dry weight) and rutin (ranging from 3.20 to 10.01 mg g^{-1} dry weight). Total phenolic levels in elderflower teas were 23.98 ± 0.838 ug g^{-1} and increased by 47% over a 20 min infusion time. Volatile profiles were measured in fresh and dried flowers and in teas made from these flowers. One of the prevalent compounds in fresh flowers and tea made with fresh flowers that appears unique to this subspecies is methyl eugenol (16.90% and 20.14% of relative peak area, respectively). Drying the flowers significantly changed the headspace volatile profile. Levels of methyl eugenol were reduced to 2.46% of the relative peak area whereas 3-hexen-1-ol levels were increased. Tea made from the fresh and dry flowers had relatively high levels of straight-chain aldehydes as compared to the flowers. Elderflowers of *S. nigra* ssp. *cerulea* can be used to make differentiated elderflower products for consumers interested in bioactive compounds and unique sensory profiles.

Keywords: phenolic composition, aroma compounds, post-harvest processing, herbal tea

1. Introduction

The elderberry (*Sambucus nigra* L.*)* is a deciduous, multi-stemmed shrub or small tree.[2,14] It can grow several meters high and in diameter and produces hundreds of clusters of aromatic flowers (i.e., elderflowers) in the spring, that mature into small berries in summer. The plant grows well in a variety of soils and climates, and is a native of Northern America, Europe, and parts of Asia.[2,14] While there are many subspecies within *Sambucus nigra*, the primary subspecies widely grown and commercially cultivated include *S. nigra* ssp. *nigra* found across Europe, and the "American" subspecies *S. nigra* ssp. *canadensis*, which is native to the eastern regions of North America.[56] The blue elderberry (*S. nigra* ssp. *cerulea*), is a drought-tolerant subspecies native to the western region of North America. The blue elderberry grows in riparian ecosystems from southern British Columbia, Canada to northwest Mexico.[84] In California, there have been efforts for more than a decade to increase the levels of blue elderberry planted in hedgerows on farms because of its environmental benefits, such as improving the air, water, and soil quality, as well as providing food and shelter for pollinators.[111] It is now recognized that these mature hedgerow plants can be a source of locally grown elderberries and elderflowers to increase income and sustainability for the farm. However, to date there is no data on the concentration of the aroma or phenolic compounds in the flowers from this hardy heat-tolerant subspecies.

The berries, flowers and bark of the elderberry plant have a long history of use by humans as both food and traditional medicine.[112-9] Seeds have been found in archeological sites that date to the late stone age (~4,000 B.C) and their medicinal use is documented in the writings of Theophrastus (371-287 B.C.), Pedanius Dioscorides (40-90 A.D.) and Gaius Plinius Secundus (23-70 A.D.).[112] Elderflowers are frequently used in medicinal and herbal teas, tonics, liqueurs, lemonades, and sparkling waters for their subtle and unique floral, fruity, and green aromas and

medicinal properties. Infusions of elderflowers (teas) have been used in many cultures for the treatment of inflammation, colds, fever, and respiratory illness and for their diuretic and antidiabetic effects.[113] Some studies have found evidence to support their use, such as antimicrobial activity of elderflower extract against Gram-positive bacteria and high *vitro* antioxidant activity.[29,114] Much of the interest for using elderflower in health-promoting applications is based on the high content of biologically active phenolic compounds in the flowers.

European and American elderflowers contain an array of phenolic compounds, such as phenolic acids (chlorogenic acids), flavonols (kaempferol, quercetin), flavonol glycosides [isorhamnetin-3-*O*-rutinoside (IR), rutin (quercetin-3-*O*-rutinoside)], flavan-3-ols [(+)-catechin, (-)-epicatechin], and flavanones.[6,114,115] In European-grown elderflowers, the dominant phenolic acid and flavonol glycoside include chlorogenic acid and rutin, although isoquercetin, isorhamnetin-3-rutinoside and kaempferol-3-rutinoside are also present.[6,71,114,115] For example, in a study of European elderflowers grown in different locations and altitudes, the dominant class of phenolic compounds were the flavonols, namely rutin (0.31362 to 1.65490 mg g^{-1}), whereas chlorogenic acid levels were lower (0.20917 to 0.47025 mg g^{-1}).[6] This study also found that the flowers contain four times more chlorogenic acid than the leaves or berries. The predominant phenolic compounds identified in elderflower syrup, a traditional herbal beverage, include chlorogenic acid (1.3265-5.10873 mg g^{-1}) and rutin (0.79237-5.21695 mg g^{-1}).[115] There has been only one study on the phenolic profile of the flowers of *S. nigra* ssp. *canadensis* which appears to be similar to the European subspecies, in that rutin and chlorogenic acid are the primary flavonol and phenolic acid identified, respectively.[56]

The aroma of the elderflower is derived from the volatile organic compounds (VOCs) in the flower and is an important characteristic to understand for consumer acceptance in applications.

To date, only the VOCs of elderflowers from the European subspecies have been studied. The American subspecies *S. nigra* ssp. *canadensis* has not yet been investigated. As fresh flowers are highly perishable, many commercial products rely on dry, and in some cases, frozen flowers. Thus, it is important to understand how the organoleptic properties of elderflowers change in response to processing. The VOC profile of tea made with elderflowers of three European cultivars using dynamic headspace sampling revealed compounds important to the characteristic aroma to be linalool, hotrienol, and *cis*- and *trans*-rose oxide.[75] Similarly, studies indicate that in fresh and dried flowers analyzed by headspace solid phase microextraction coupled with gas chromatography mass spectrometry (HS-SPME/GC-MS), linalool oxides are the main aroma compounds.[76,77] Linalool oxide has a floral, herbal, earthy, green odor.[80] In hexane extracts of dry elderflowers analyzed via HS-SPME/GC-MS, *cis*-linalool oxide and 2-hexanone were the primary volatiles.[78] The compound 2-hexanone has a fruity, fungal, meaty, and buttery odor.[80] In syrups made from elderflowers, terpene alcohols and oxides were identified as the primary aroma compounds.[79] Studies of the impact of drying on volatiles in the flowers demonstrate that nearly all types of drying (i.e. air drying, freezing, freeze-drying, and vacuum packing) change the volatile profile significantly.[76,77]

The aim of this study was to characterize the composition of phenolic compounds and VOCs in flowers of the blue elderberry (*S. nigra* ssp. *cerulea*), and to determine how these compounds change in response to drying and in the preparation of teas. Understanding how the aroma and phenolic compounds compare with current commercially available European and American subspecies will help to establish a role for blue elderflowers in commercial applications such as herbal teas and as a flavoring for beverages, as well as identify unique compositional qualities of this native and underutilized flower.

2. Materials and methods

2.1. Chemicals

LC/MS-grade acetonitrile and HPLC-grade hydrochloric acid (HCl, 12 N) were purchased from Fisher Scientific (Hampton, NH). HPLC-grade ethanol and acetonitrile were purchased from Sigma Aldrich (St. Louis, MO). Purissimum grade phosphoric acid (85%) was purchased from Sigma Aldrich (St. Louis, MO) and filtered through 0.45 µm polypropylene filters under vacuum. Ascorbic acid was obtained from Acros Organics (Fairlawn, New Jersey). Ultrafiltered water (18.2 MΩ) was obtained by a Milli-Q system (Millipore, Sigma Aldrich, St. Louis, MO). Analytical standards of rutin, quercetin, chlorogenic acid, and (+)-catechin were purchased from Sigma Aldrich (St. Louis, MO). A standard of n-butyl-d_9 was purchased from CDN Isotopes (Quebec, Canada). Kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-glucoside, IR, and isoquercetin were purchased from ExtraSynthese (Genay, France).

2.2. Plant material

Elderflowers were harvested from hedgerows on a farm in Winters, CA in May and June 2021. The latitude and longitude coordinates of the hedgerow are 38.634884, -122.007502. Flowers were harvested between 8 and 10 am and were picked from all sides of the shrub. Picked flowers were placed in plastic bags, immediately put on ice, and transported to the laboratory at the University of California, Davis. Flowers were either dried at 25 °C for 24 h in a dehydrator (Tribest Sedona Express, Anaheim, CA, United States) or analyzed fresh. Once dry, stems were removed, and flowers were stored in oxygen-impermeable aluminum pouches. Triplicate samples of fresh flowers were analyzed for their moisture content by drying 1 g of fresh flowers at 95 °C

(Sartorius, Göttingen, Germany) until a consistent weight was achieved so that the same amount of dry matter could be used for fresh and dry flower analyses.

2.3. Phenolic compound analysis

2.3.1. Sample preparation

An aqueous mixture of ethanol was used to extract the phenolic compounds from flowers. The optimal mixture of ethanol to water (v/v) was determined by extracting flowers in 0, 25, 50, 75, and 100% ethanol. Solvents also contained 0.1% HCl and 0.1% ascorbic acid (v/v). For each extraction, 0.25 g dry flower material and 25 mL solvent were added to 50 mL Eppendorf tubes. The dry flowers with solvent were homogenized for 1 min at 7000 rpm with a 19 mm diameter probe head (IKA, Wilmington, NC) in the 50 mL tubes. Homogenized extracts were refrigerated overnight at 4 °C, then centrifuged at 4000 rpm (3005 rcf) for 7 min (Thermo Scientific, Waltham, MA). The supernatant was filtered through 0.45 µm PTFE, then diluted 50% (v/v) with 1.5% phosphoric acid before analysis. Three replicates were made for each extraction condition (n = 3).

Phenolics were extracted from fresh and dried flowers that were either whole or homogenized. Hence, four types of samples were made: fresh whole flowers (FWF), dry whole flowers (DWF), fresh homogenized flowers (FHF), and dry homogenized flowers (DHF). Flowers were mixed with the determined optimal extraction solvent and followed the same extraction process as described above, except whole flower samples were not homogenized and instead placed directly into the refrigerator to extract overnight.

2.3.2. Preparation of infusions (tea)

To understand the phenolic extraction in hot water, elderflower tea samples were prepared by combining 2.0 g of dry flowers with 240 mL of boiling water, then stirred for 10 s. These parameters mimic the consumer experience of making a cup of hot tea with dry elderflower, typically about a tablespoon. Aliquots of tea were removed at 5, 10, 15, and 20 min. Three replicate batches of tea were made (n=3).

2.3.3. Quantitation of phenolic compounds by HPLC-DAD-FLD

All sample extracts were analyzed via high performance liquid chromatography (HPLC) using an Agilent 1200 system with diode array detection (DAD) and fluorescence detection (FLD) (Santa Clara, CA). Separation of phenolic compounds was performed on an Agilent PLRP-S column (4.6 mm x 150 mm, 3 µm) at 35 °C, using a previously published method.[116] Mobile phase A was 1.5% phosphoric acid in water and mobile phase B was 80% acetonitrile, 20% mobile phase A (v/v). The flow was set at 1.00 mL min^{-1}. The gradient used was as follows: 0 min, 6% B, 73 min, 31% B, 78-86 min, 62% B, 90-105 min 6% B. Most phenolic compounds were detected using a (DAD) at 280 nm (hydroxybenzoic acids and simple phenols), 320 nm (hydroxycinnamic acids), and 360 nm (flavonols). Flavan-3-ols were detected using a fluorescence detector (230 nm excitation, 321 nm emission). Compounds were quantified using external standard curves employing surrogate standards for each group of phenolic compounds [(+)-catechin for flavan-3-ols, chlorogenic acid for phenolic acids and simple phenols, quercetin for flavonol aglycones, and IR for flavonols]. Standards were prepared at concentrations of 200, 100, 50, 10, and 5 mg L^{-1}, except IR which included an additional concentration of 500 mg L^{-1}. Triplicate analyses of each concentration were performed (n=3).

2.3.4. Confirmation of phenolic compounds by HPLC-QTOF-MS/MS

Compounds were separated using HPLC-DAD-FLD as described above and identified using authentic standards to check retention time (t_R) and absorption spectra. Several peaks in the chromatograms did not match t_R or spectra of authentic standards. Therefore, fractions of these peaks were collected. Fractions were dried and reconstituted in 1% formic acid in water. These samples were then subjected to high resolution mass spectrometry using an Agilent 6545 quadrupole time-of-flight mass spectrometer (QTOF-MS/MS), using conditions previously established for elderberry phenolic compounds.[39] Data were then analyzed using Agilent MassHunter Workstation Qualitative Analysis 10.0 (Agilent, Santa Clara, CA, USA). To tentatively identify compounds, the mass to charge (*m/z*) ratio of the precursor and fragment ions were compared to online libraries of compounds and using formula generation for the peaks in the spectra.

2.4. Volatile analysis by HS-SPME/GC-MS

Volatile compounds were analyzed by headspace solid phase microextraction gas chromatography mass spectrometry (HS-SPME/GC-MS). The equilibration and extraction parameters were optimized using ground dry flowers, prepared using a spice grinder, pulsed 25 times (Waring, Stamford, Connecticut). A 1 g sample of ground dry flowers was placed into a 20 mL glass vial and the vial was sealed by a crimp-top cap with a Teflon septa.

Various incubation temperatures (30, 40, 50, 60 °C), equilibration times (10, 20, 30, 40 min), and extraction times (10, 20, 30, 40, 60, 75, 90 min) were evaluated to optimize for the highest total peak area and unique compounds identified from samples. The fiber used for all analyses was a divinylbenzene/carbon wide range/polydimethylsiloxane (DVB/CAR/PDMS), 23 Ga, 1 cm length, with 80 µm phase thickness (Agilent, Santa Clara, CA). After extraction, the fiber

was injected into the GC (7890A, Agilent, Santa Clara, CA) and volatile compounds were desorbed at 250 °C for 5 min. Compounds were then separated on a DB-Wax column (30 m, 0.25 mm i.d., 0.25 µm film thickness) (Agilent, Santa Clara, CA). Helium was used as a carrier gas at 1 mL min^{-1}. A temperature program was used with the following steps: 35 °C for 1 min, 3 °C min^{-1} to 65 °C, 6 °C min^{-1} to 180 °C, 30 °C min^{-1} to 240 °C, hold at 240 °C for 5 min. Total run time was 37.167 min. Compounds were detected with a single quad, triple axis mass spectrometer (5975 C, Agilent, Santa Clara, CA). The mass range for acquisition was 30 to 300 m/z. The MS transfer line temperature was 250 °C, the source temperature was 230 °C, and the quad temperature was 150 °C. The electron ionization was set to 70 eV.

To have the same volume of headspace in fresh and dry flower samples, 0.5 g of fresh whole flowers or 1.5 g ground dry flowers were placed in the 20 mL clear glass vials. For tea samples, 4 mL tea was placed in 20 mL vials. To each sample, 10 µl of 1-butanol-d9 in methanol (2 mg L^{-1}) was added as an internal standard. Volatile compounds were identified using Agilent Mass Hunter Unknown Analysis (Santa Clara, CA, version B7.00), using the NIST17 library (National Institute of Standards and Technology, Gaithersburg, MD) requiring an ≥ 80% match and that compounds were identified in at least three of the five to be considered a volatile compound in the samples. An alkane series (C9 to C18) was run under the same chromatographic conditions to determine retention indices. Confirmation of identification was performed by comparing the mass spectra and retention indices with those of standards when possible or literature values when standards were not accessible. Relative response was calculated by normalizing peak area for each compound to the internal standard peak area, and relative peak area was calculated using the relative response of a compound divided by the total peak area of a sample.

2.5. Statistical analysis

Statistical analysis was performed in Microsoft Excel (Redmond, WA) and RStudio (R Core Team, Boston MA). Two-way ANOVAs were run on phenolic concentrations in different extractions conditions. Tukey's Honestly Significant Differences were calculated with a p value of 0.05. Average peak area of volatile compounds and relative peak areas were calculated using Microsoft Excel.

3. Results and discussion

3.1. Analysis of phenolic compounds

The phenolic compounds were measured in fresh and dry elderflowers of *S. nigra* ssp. *cerulea*, both as whole and as homogenized flowers. The treatments used for this study were chosen to reflect the common ways that elderflowers are used in food and beverage applications and to provide more information on how to best extract the phenolic compounds from the flowers. The moisture content of the elderflowers was determined as 75.6 ± 1.7%. To achieve a consistent dry weight used in extractions, either 1.00 g of fresh flowers or 0.25 g of dry flowers were used. The extraction solvent was optimized to increase extraction efficiency of the main phenolic acids, flavonols and flavan-3-ols which included chlorogenic acid, IR, rutin, and (+)-catechin (Figure 1). While chlorogenic acid, rutin, and catechin could be extracted in either 50:50% ethanol:water (v/v) or 25:75% ethanol:water (v/v) for maximum concentrations, the levels of IR increased with increasing amounts of water in the solvent system. However, in solvents containing ≥ 75% water, the flowers turned brown in color suggesting extensive oxidation. Therefore, it was determined that 50:50% ethanol:water (v/v) was the optimal solvent for the extraction of the range of phenolic compounds in elderflowers without excess oxidation. A recent study of the effect of organic

solvents (methanol, ethanol, or acetone) on the extraction of phytochemicals from butterfly pea flowers also found that 50:50% ethanol:water (v/v) had optimal extraction properties for the phenolic compounds in flowers.[117] These results differ from a study on the extract of phenolic compounds from dry, powdered European elderflower, which found water to be the optimal extraction solvent, specifically at 100 °C for 30 mins, as compared to 80:20% ethanol:water (v/v) or 80:20% methanol:water (v/v).[71]

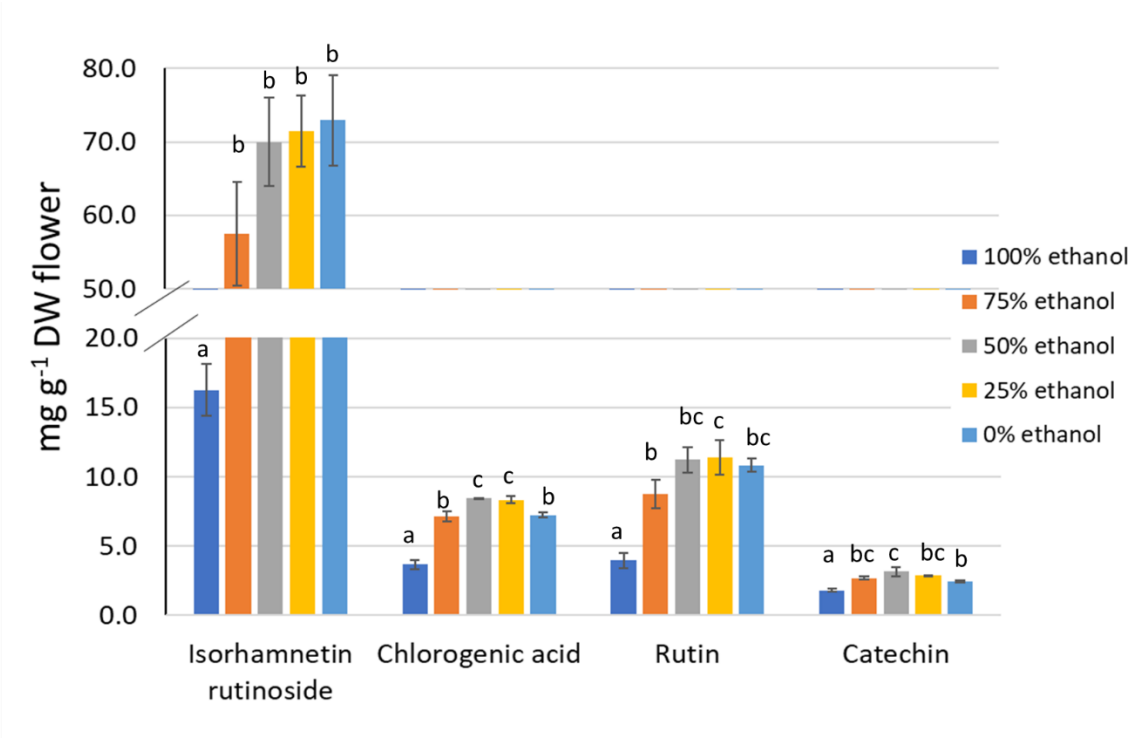


Figure 1. Concentrations of phenolic compounds in dry flowers blended with 0-100% ethanol in water in mg g^{-1} dry flower.

Elderflowers are used in products as either fresh or, more commonly, as dry flowers and as either whole or homogenized flowers (powder). Therefore, each of these parameters were evaluated resulting in the following types of samples: fresh whole flowers (FWF); dry whole flowers (DHF); fresh homogenized flowers (FHF); dry homogenized flowers (DHF). Phenolic

compounds were quantified using HPLC-DAD-FLD and information regarding the standard curves can be found in Table 1. Significantly more phenolic compounds (an approximate two-fold increase) were extracted from FHF compared with FWF, DWF and DHF (Table 2) indicating that phenolic compounds are released more readily from the vacuoles during homogenization while the flower is still fresh.[118] There was no significant difference in the sum of all measured phenolic compounds between FWF, DWF, or DHF; however, levels of most phenolics were slightly higher in the DHF, suggesting that homogenization also increases the extraction efficiency in dry flowers. Furthermore, a statistically significant interaction was found between the fresh and dry flowers and homogenization of the sample for most phenolic compounds, due to the uniquely high levels present in the FHF and the absence of an equally high increase in DHF (Table 2). This trend can also be seen in the totals of each phenolic class (flavan-3-ols, acids, flavonols, and total phenolic compounds), as the FHF were significantly higher than all other sample types, with the exception of total flavan-3-ols in DHF (Table 2).

Table 1. Calibration curve linear equations, correlation coefficients, lower limits of detection (LLOD) and quantitation (LLOQ).

Compound	Linear equation	R^2	LLOD (mg g^{-1})	LLOQ (mg g^{-1})
Chlorogenic acid	y = 31.102x – 100.59	0.9994	0.63	1.90
Catechin	y = 29.223x – 33.461	0.9999	1.52	4.33
Isorhamnetin-rutinoside	y = 14.028x – 20.258	1.0000	1.11	5.72
Quercetin	y = 44.410x – 143.22	0.9997	0.00	0.00

LLOD and LLOQ were calculated as 3.3 times and 10 times, respectively, the standard deviation of the intercept divided by the slope of the standard curve.

Table 2. Concentration (mg g^{-1} dry weight) of phenolic compounds in different elderflower preparations (average ± SD, n = 3).

Phenolic compounds	FHF	FWF	DHF	DWF	Interaction of flower:treatment
Proanthocyanidin B type [a]	0.39 ± 0.04 a	0.36 ± 0.05 a	0.36 ± 0.01 a	0.33 ± 0.06 a	-
Catechin	1.11 ± 0.30 c	0.43 ± 0.13 a	0.79 ± 0.32 bc	0.59 ± 0.22 ab	-
Epicatechin	1.24 ± 0.18 b	0.44 ± 0.04 a	0.57 ± 0.14 a	0.48 ± 0.09 a	***
Total flavan-3-ols	2.73 ± 0.50 b	1.24 ± 0.19 a	1.72 ± 0.53 ab	1.40 ± 0.33 a	*
5-HPG hexoside	2.00 ± 0.72 b	1.18 ± 0.33 a	1.42 ± 0.18 ab	1.26 ± 0.20 a	-
Coumaroyl-caffeoylquinic acid isomer [b]	5.18 ± 0.81 b	2.96 ± 0.29 a	2.83 ± 0.30 a	2.51 ± 0.39 a	**
Caffeoylquinic acid isomer [c]	1.79 ±0. 28 b	1.32 ± 0.42 a	1.12 ± 0.15 a	1.14 ± 0.34 a	*
Caffeoylquinic acid isomer [c]	1.55 ± 0.10 c	1.14 ± 0.13 b	0.99 ± 0.11 a	1.07 ± 0.44 b	***
Neochlorogenic acid	1.10 ± 0.13 b	0.90 ± 0.51 ab	0.84 ± 0.06 a	0.77 ± 0.17 a	-
Chlorogenic acid	10.36 ± 0.25 c	4.12 ± 0.19 a	5.61 ± 1.07 b	4.36 ± 0.28 ab	***
Total phenolic acids	19.97 ± 1.30 b	10.43 ± 0.39 a	11.40 ± 1.62 a	9.85 ± 0.62 a	***
Quercetin-3-*O*-rutinoside (rutin)	10.01 ± 0.98 c	3.20 ± 0.40 a	6.26 ± 0.61 b	4.84 ± 0.35 ab	***
Kaempferol-3-*O*-rutinoside	3.01 ± 0.37 b	1.24 ± 0.03 a	1.35 ± 0.02 a	1.34 ± 0.36 a	***
Isorhamnetin-3-*O*-rutinoside	78.73 ± 4.84 b	32.48 ± 3.41 a	34.40 ± 2.07 a	33.64 ± 8.81 a	***
Isorhamnetin-3-*O*-glucoside	1.42 ± 0.40 b	0.92 ± 0.09 a	0.77 ± 0.00 a	0.78 ± 0.23 a	*
Quercetin	2.41 ± 0.25 c	2.32 ± 0.16 bc	1.90 ± 0.065 a	1.57 ± 0.46 ab	-
Total flavonols	95.58 ± 3.15 b	40.17 ± 2.69 a	44.69 ± 1.39 a	42.17 ± 10.29 a	***
Total Phenolic Compounds	120.28 ± 3.97 b	53.02 ± 2.81 a	59.21 ± 1.95 a	54.68 ± 9.95 a	***

fresh whole flowers (FWF); dried whole flowers (DHF); fresh homogenized flowers (FHF); dried homogenized flowers (DHF). [a] tentative identification via LC/QTOF-MS, *m/z* 577.1340, 425.0874, 289.0718. [b] tentative identification via LC/QTOF-MS, m/z 499.1461, 353.0875, 191.0556, 173.0466. [c] tentative identification via LC/QTOF-MS, m/z 353.0880, 191.0564. Concentrations for a compound or class in a row that share a letter are not significantly different ($p < 0.05$) based on Tukey's HSD. For interaction of flower (fresh or dry) by homogenized (whole or homogenized), * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, - means not significant.

The most abundant phenolic compound found in extracts of the blue elderflowers was IR. The levels of IR were significantly higher in FHF, with a maximum concentration of 78.73 ± 4.84 mg g^{-1}. This is a significant difference as compared with the European and American subspecies, in which rutin is the predominant phenolic compound in flowers and at much lower concentrations.[6,56,74,115,119] Levels of IR in European elderflower levels range from about 0.200 to 0.900 mg g^{-1} fresh weight,[6,115] though higher levels were found in elderflower tea, ranging from 4.260 to 13.500 mg g^{-1}.[119] This key difference in the flowers of the blue elderberry provides an opportunity to create unique products for consumers looking for high levels of bioactive phenolic compounds, as studies have shown that IR can induce apoptosis in cancer cells.[120,121]

The other flavonol glycosides found in the flowers include rutin, kaempferol-3-*O*-rutinoside, and isorhamnetin-3-*O*-glucoside. Rutin ranged from 3.20 ± 0.395 µg g^{-1} in FWF to 10.01 ± 0.97 mg g^{-1} in FHF (Table 2). The concentrations of rutin vary greatly across studies of European elderflowers. For example, levels are reported as 1.65490 ± 0.10951 mg g^{-1} fresh weight,[6] 1.8877 ± 0.2691 mg g^{-1} fresh weight,[114] between 15.70 and 23.90 mg g^{-1} in dried elderflower tea which varied with cultivar,[119] and 4.14443 ± 0.11469 mg g^{-1} in a traditional beverage called sabesa which is made from fresh flowers.[115] Herein, kaempferol-3-*O*-rutinoside ranged from 1.24 ± 0.03 to 3.010 ± 0.37 mg g^{-1} dry weight, and isorhamnetin-3-*O*-glucoside ranged from 0.77 ± 0.05 to 1.42 ± 0.40 mg g^{-1} dry weight (Table 2). Quercetin was the only flavonol aglycone identified in the flower extracts and was low relative to the flavonol glycosides. Though this compound may be due to the degradation of a quercetin glycoside, quercetin aglycone has been measured in other elderflower studies, and our results are similar to those reported by Viapiana et al. (2017).[122] The flavan-3-ols monomers found in the flowers include (+)-catechin and (-)-epicatechin, highest in the FHF at 1.110 ± 0.30 and 1.24 ± 0.19 mg g^{-1}, respectively (Table

2). (-)-Epicatechin, but not (+)-catechin, had an interaction between the fresh and dried and homogenization of the sample, as it was significantly higher in FHF. Proanthocyanin B type was also tentatively identified via HPLC-(ESI)MS/MS analysis in the flowers, and was present in relatively low quantities in all samples (Table 2). A procyanidin trimer was identified in elderflowers extracts and beverages by Mikulic-Petkovsek et al. (2015).[115]

Chlorogenic acid (3-*O*-caffeoylquinic acid) was identified as the main phenolic acid in the flowers of the blue elderberry, like the flowers of the American elderberry,[56] whereas the predominant phenolic acid in the flowers of the European elderberry is neochlorogenic acid (5-*O*-caffeoylquinic acid).[74,115,119] Neochlorogenic acid and other caffeoylquinic acid isomers were also present in the elderflowers of *S. nigra* ssp. *cerulea* (Table 2). Two isomers of 5-caffeoylquinic acid in addition to 3- and 4-caffeoylquinic acid have been identified in elderflower products.[115] Evaluation of the phenolic content of elder plants grown in different locations and altitudes indicate, in general, that plant material from shrubs at higher altitudes had higher levels of hydroxycinnamic acids and flavonols.[6] The authors postulated that the stress of harsher climates at higher altitudes may have led to the increase in hydroxycinnamic acids and flavonols to cope with the increase in UV radiation. They also reasoned that the high amounts of sun and cool nights may increase the metabolism of phenolic compounds. The flowers in the present study experience hot, dry summers with cool breezes from the Sacramento-San Joaquin Delta at night, and these conditions may contribute to the unique phenolic profile in this flower. The average day/night temperatures for Davis, California while the flowers were growing were 24/7 °C in April 2021, 28/12 °C in May 2021, and 31/13 °C in June 2021, with less than 3 inches of rain during that time span.[123]

A phenolic compound unique to the blue elderflower was identified as 5-hydroxypyrogallol (5-HPG) hexoside ($C_{12}H_{16}O_9$, exact mass = 304.0794). This compound has also been identified in the berries of this subspecies (21.50 ± 18.32 mg 100 g^{-1} fresh weight) grown in the same locations.[39] The concentrations of 5-HPG ranged from 1.26 ± 0.20 mg g^{-1} in DWF to 2.00 ± 0.72 mg g^{-1} in FHF (Table 2). Because no commercial standards exist for this compound, the tentative identification of this compound was determined by high resolution QTOF-MS/MS data. The mass spectrum (Figure 2) shows the molecular ion $[M-H]^-$ at *m/z* 303.0728 and fragment ion showing the loss of the sugar molecule $[M\text{-hexose-H}]^-$ at m/z 141.0199. Although the biological properties of this unique phenolic compound have not yet been investigated, 5-HPG hexoside can serve as a marker for *S. nigra* ssp. *cerulea*, especially since it is present in relatively high levels in the flower and berry and is not identified in other elderberry subspecies.

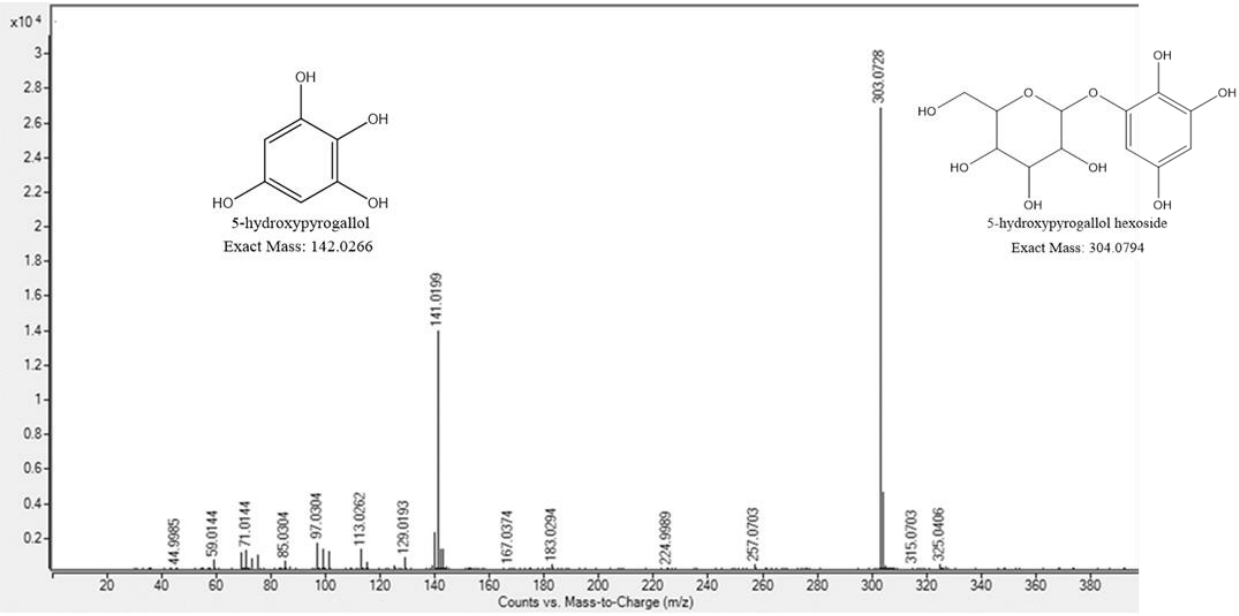


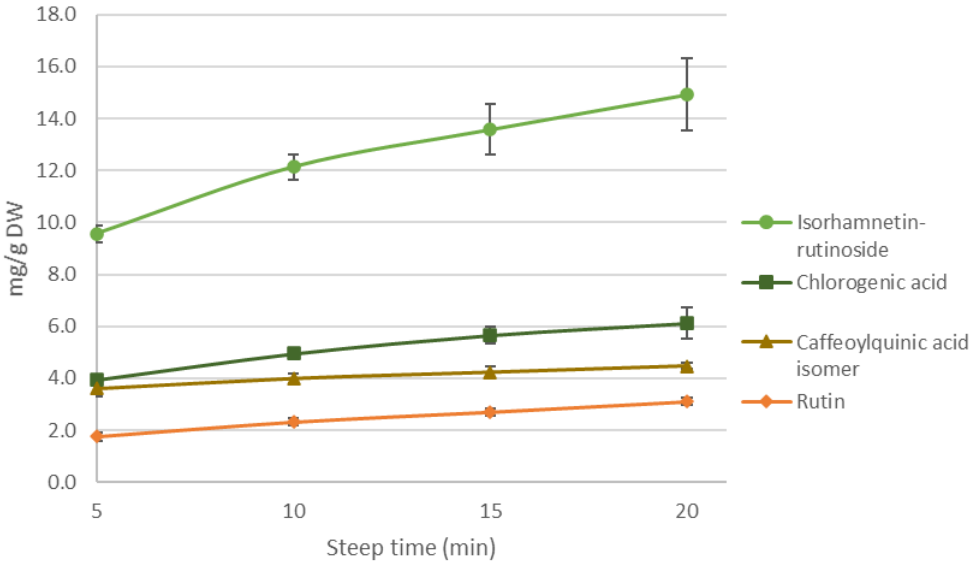
Figure 2. HPLC-QTOF-MS/MS spectra of a novel phenolic compound 5-hydroxypyrogallol hexoside.

Elderflower tea is one of the most traditional and simplest ways that the flowers are used in the preparation of beverages. To make elderflower tea, the flowers are infused in hot water

(steeped) to extract the flavor and biologically active phenolic compounds from the flowers. Recommended steeping times can vary widely, however there are no studies investigating the impact of steep time on the extraction of phenolic compounds in the elderflower tea. To address this, the impact of time on the extraction of phenolic compounds from teas made from dried flowers was evaluated. The profile of phenolic compounds extracted in hot water infusions was similar to the profile obtained in ethanol/water extracts, however the concentrations were lower in the hot water extracts (Figure 3). Phenolic compounds were quantified at 5, 10, 15, and 20 minutes of steep time. Over time, the concentrations of total measured phenolic compounds increased 47% from five minutes (total phenolic concentration of 23.98 ± 83.78 mg g^{-1} to 20 minutes (total phenolic concentration of 35.22 ± 2.38 mg g^{-1}). These results suggest that longer infusion times are beneficial for extracting the highest level of compounds.

The overall levels of phenolic compounds in elderflower of *S. nigra* ssp. *cerulea* are comparable to the levels in the European flowers. However, variability between studies due to post-harvest conditions, extraction solvent, and analytical method make it challenging to make direct comparisons. For example, in this study, FWF, DWF, and DHF all had about 50-60mg g^{-1} dry weight, while the FHF had ~120 mg g^{-1} dry weight. The unique composition of phenolic compounds in these flowers provides an opportunity to make different products for the market, especially nutraceutical or functional food products that take advantage of the high levels of IR. Based on our results, it would be best to use fresh flowers and blend or homogenize the flowers to extract the highest levels of phenolic compounds.

(a)



(b)

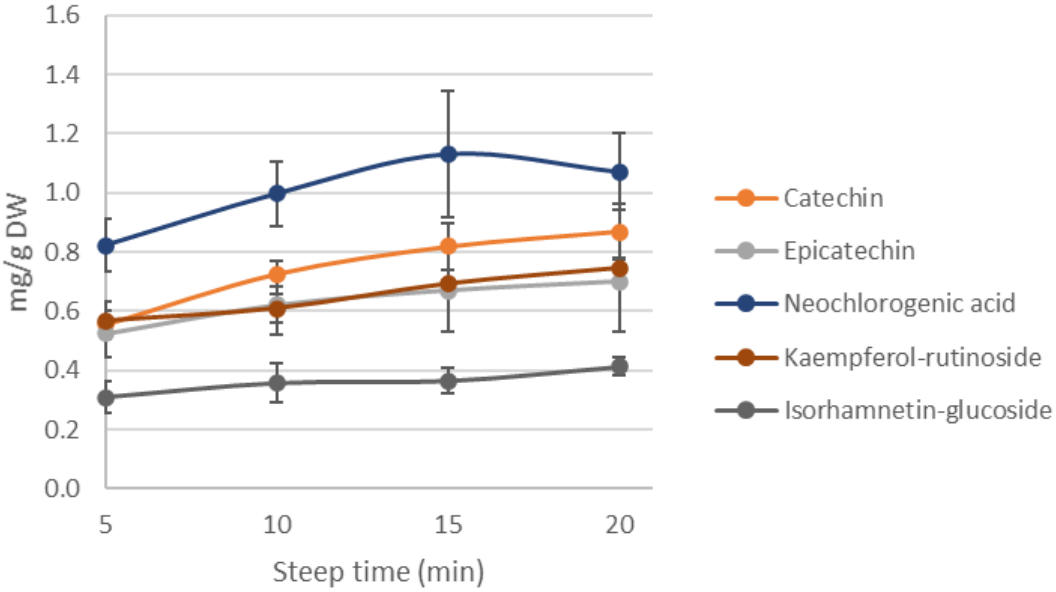


Figure 3. Concentrations of phenolic compounds (a) Compounds > 1.50 mg g^{-1} dry flower (b) Compounds < 1.50 mg g^{-1} dry flower) in hot water infusions (teas) of dry flowers throughout steep time (n = 3).

3.2. Analysis of the volatile profile

Before analyzing samples, method parameters were evaluated to find the optimal equilibrium time, temperature, and the extraction time for headspace VOCs. The optimal parameters for flower samples were determined to be 40 °C, 20 min equilibration (with agitation at 500 rpm), and 30 min extraction with a SPME fiber. The optimized conditions for the tea samples were 40 °C, 30 min equilibration, and 30 min extraction. The profiles of volatile compounds were evaluated in fresh whole, dry ground, and teas made from whole fresh and dry flowers by HS-SPME/GC-MS, and relative levels of identified volatile compounds were calculated. The fresh flowers were evaluated as whole flowers because homogenizing them can cause oxidation and create artifacts in the volatile headspace profiles. This led to higher variability in the relative peak areas of compounds, but it is believed to be truer to the real headspace VOC profile as compared to a homogenized fresh flower sample. Overall, 25 compounds in the fresh whole flowers, 44 compounds in the dry ground flowers, and 18 compounds in the tea preparations were identified. Table 1S contains the *m/z* ion of the base peak and the average match factor (%) for each of the compounds identified in the headspace of samples.

In the headspace of fresh flower, the most concentrated compounds (relative area > 5%) were pentadecane > methyl eugenol > *cis*-3-hexenyl acetate > α-farnesene > and *cis*-3-hexenyl-α-methylbutyrate (Table 3). The contribution of each compound's odor to the overall aroma of these elderflowers cannot be determined from the concentration alone, as each compound has its own odor activity and threshold.[124] However, it can be useful to know the characteristic odors of these compounds as a way to understand what comprised the general aroma. These compounds are described to have odors such as waxy; clove, spice; fresh, green, sweet, fruity, apple, pear, melon;

wood, sweet; ; fruity, sweet, minty, fresh, and green apple, respectively.[80,125,126] In addition to pentadecane, several straight chain hydrocarbons were also present, which may be released from the cuticle of the petal or peduncle of the flower.[127] These include 1-pentadecene, heptadecane, 8-heptadecene and 6,9-heptadcadiene. Flowers also contain 4.6% methyl salicylate (oil of wintergreen) a compound with a sweet, minty odor[80] that is frequently used as an analgesic in liniments to relieve pain. Methyl salicylate has been identified in several other studies on the volatile profile of elderflowers.[76,81,128] The profile of headspace VOCs in elderflowers of *S. nigra* ssp. *cerulea* differ significantly from the European elderflowers (the only other subspecies that has been evaluated for VOCs) as linalool oxides and other derivatives predominate in the European flowers,[75–79] and are absent in the present study. Furthermore, the present study indicates a unique headspace VOC profile in the blue elderflowers because pentadecane and methyl eugenol have been identified as major contributors to the headspace VOC profile. Pentadecane has been identified at trace levels in some European elderflower extracts,[76,129] however methyl eugenol has not been identified in European elderflowers. Methyl eugenol, which has a clove-like aroma[19], appears to be unique to the *S. nigra* ssp. *cerulea* elderflower, and could be a unique volatile marker for this subspecies.

Tea made with fresh whole elderflowers presented a slightly different headspace VOC profile as compared to fresh flowers. Although methyl eugenol was still a prominent compound in the headspace (20.14 ± 3.06%), there were also two ketones in relatively high concentrations: 2,2,6-trimethyl-4H-1,3-dioxin-4-one (13.78%) and 4-methyl-2-heptanone (12.30 ± 2.51%) (Table 3). Aroma descriptors were not found for these compounds. In addition, two aldehydes including heptanal (17.68 ± 1.34%) and nonanal (14.69 ± 0.78%) were also present in the headspace of the tea but not the fresh flowers. Heptanal odor is described to be fresh, fatty, green, and herbal,

whereas nonanal is described to have waxy, rose, orange peel or fatty notes.[80] Methyl salicylate comprised only 1.27 ± 0.29% of the headspace volatiles in the tea made from fresh flowers.

In the headspace of dry elderflowers, the most concentrated compounds (relative area > 5%) were (*Z*)-3-hexen-1-ol > 1-penten-3-ol > 3-methyl-butanal > heptanal, > isocyanato-methane (Table 4). In general, the dry flowers contain a wider range of volatiles than the fresh flowers, including more aldehydes, alcohols, alkanes, and other hydrocarbons. Other notable volatiles identified include methyl salicylate (2.96 ± 0.15), dihydroedulan (0.21 ± 0.12%), which is a driver of typical elderberry (not elderflower) aroma,[63] and linalyl acetate (0.26 ± 0.02%) which is the only linalool derivate identified any preparations of the elderflowers of *S. nigra* ssp. *cerulea*, unlike European elderflowers which typically have high concentrations of linalool derivatives.[75–79]

In tea prepared from dried flowers, the headspace aroma less complex than the dried flowers, but many of the aldehydes were still identified, including nonanal (23.62 ± 1.55%), heptanal (21.22 ± 0.59), and hexanal (9.32 ± 1.01%) (Table 4). Levels of methyl salicylate were about two-fold higher that fresh flower tea (2.90 ± 0.41%). In addition, methyl eugenol, an important compound in fresh flowers that has not been identified in European elderflowers was also present in low quantities (2.46 ± 0.66%), meaning aqueous products that use dry flowers may have a unique aroma profile as compared to the European elderflower that are used in virtually all commercial elderflower products.

Table 3. Relative peak area of headspace volatile compounds in fresh whole elderflowers (n = 5) and in tea prepared with fresh whole (n = 3) elderflowers (avg ± SD %).

Compound	RI[a]	Relative peak area (%) in fresh flowers	Relative peak area (%) in tea	Odor
2,4-dimethyl-1-heptene	n/a[c]	-	0.33 ± 0.16	----
n-butyl ether	948	-	0.11 ± 0.06	ethereal[e]
hexanal[b]	1035	-	3.53 ± 0.24	green, grassy[d]
o-formic acid, triisobutyl ester	1109	-	0.05 ± 0.02	----
heptanal[b]	1142	-	17.68 ± 1.34	fresh, fatty, green, herbal[e]
4-methyl-2-heptanone	1161		12.30 ± 2.51	----
D-limonene[b]	1162	4.58 ± 0.27	-	citrus orange fresh sweet[e]
2,2,6-trimethyl-4H-1,3-dioxin-4-one	1198	-	13.78 ± 0.31	----
hexyl acetate[b]	1230	3.39 ± 1.46	-	----
octanal[b]	1237	-	3.24 ± 0.04	citrus-like, green[d]
cis-3-hexenyl acetate[b]	1265	10.37 ± 1.70	-	fresh, green, sweet, fruity, apple, pear, melon[e]
1-hexanol[b]	1286	0.54 ± 0.08	1.97 ± 0.07	ethereal fusel oily fruity alcoholic sweet green[e]
(*Z*)-3-hexen-1-ol[b]	1307	3.51 ± 0.41	-	fresh green cut grass foliage vegetable herbal oily[e]
4-methyl-2-oxo-pentanoic acid methyl ester	1333	0.71 ± 0.16	-	----
2-methyl-propanoic acid 3-hexenyl ester	1347	0.72 ± 0.07	-	----
nonanal[b]	1349	-	14.69 ± 0.78	waxy, rose, orange peel, fatty[e]

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butanoic acid hexyl ester	1382	0.27 ± 0.07	-	green sweet fruity apple waxy soapy[e]
L-leucine methyl ester	1406	2.30 ± 0.99	2.86 ± 0.57	----
(*Z*)-butanoic acid-3-hexenyl ester	1419	4.06 ± 0.27	-	----
cis-3-hexenyl-α-methylbutyrate	1437	7.92 ± 2.20	-	fresh, green apple, sweet, fruity, pear[e]
4,7-dimethyl-undecane	1492	-	0.87 ± 0.22	----
pentadecane[b]	1500	20.65 ± 2.35	-	waxy[e]
1-pentadecene	1512	0.59 ± 0.06	-	----
2-methyl-benzaldehyde	1545	-	0.33 ± 0.09	cherry[e]
(*Z*)-hexanoic acid 3-hexenyl ester	1607	1.11 ± 0.05	-	fruity, waxy, green, fatty, tropical, pulpy, winey, balsamic[e]
germacrene D	1662	1.18 ± 0.51	-	woody[e]
methyl salicylate	1667	4.65 ± 0.71	1.27 ± 0.29	wintergreen mint[e]
α-farnesene isomer 1	1679	0.26 ± 0.05	-	wood, sweet[f]
heptadecane[b]	1691	0.59 ± 0.11	0.70 ± 0.43	alkane[f]
α-farnesene isomer 2	1708	8.23 ± 1.75	1.50 ± 0.14	wood, sweet[f]
8-heptadecene	1714	3.97 ± 0.76	-	----
6,9-heptadecadiene	1743	0.36 ± 0.17	-	----
methyl eugenol[b]	1888	16.90 ± 5.15	20.14 ± 3.06	Clove, spice[f]
eugenol[b]	2093	5.62 ± 2.60	4.65 ± 1.62	Clove, honey[f]
(*E*)-1,2-dimethoxy-4-(1-propenyl)-benzene	2192	0.37 ± 0.10	-	----
1,2,3-trimethoxy-5-(2-propenyl)-benzene	2211	0.63 ± 0.58	-	----

[a] retention index calculated with linear alkane standard mix using the linear retention index equation. [b] Identification confirmed with authentic standard. [c] not applicable as retention index could not be calculated due to elution before the earliest alkane standard. [d]Aroma descriptor obtained from *Eur. Food Res. Techn.***2008**, *228*, 265–273. (30) [e]Aroma descriptors obtained from http://www.thegoodscentscompany.com. (16). [f]Aroma descriptors obtained from https://www.flavornet.org/flavornet.html. (31). ----- indicates that no odor descriptors could be found for this compound. – indicates that compound was not identified in the samples.

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Table 4. Relative peak area of headspace space volatile compounds in dry ground elderflowers (n = 5) and in tea prepared with dry whole (n = 3) elderflowers (avg ± SD %).

Compound	RI[a]	Relative peak area (%) in dry flowers	Relative peak area (%) in tea	Odor
isocyanato-methane	n/a[c]	5.06 ± 0.30	-	----
2,4-Dimethyl-1-heptene	n/a	-	1.55 ± 0.30	----
3-methyl-butanal	n/a	7.90 ± 3.27	-	ethereal aldehydic chocolate peach fatty[e]
pentanal	931	1.67 ± 0.31	-	almond, malt, pungent[f]
n-butyl ether	948	-	0.30 ± 0.10	----
n-butyl ether	964	0.29 ± 0.15	-	----
methyl isovalerate	980	1.63 ± 0.21	-	strong apple fruity pineapple[e]
decane[b]	1000	1.37 ± 0.86	-	alkane[f]
hexanal[b]	1035	4.60 ± 0.62	9.32 ± 1.01	green, grassy[d]
2-nitro-propane	1061	1.05 ± 0.13	-	----
1-penten-3-ol	1099	10.66 ± 0.88	-	pungent horseradish green vegetable tropical fruity[e]
o-formic acid, triisobutyl ester	1109	-	0.74 ± 0.09	----
heptanal[b]	1142	6.87 ± 1.69	21.22 ± 0.59	fat, citrus, rancid[f]
3-methyl-1-butanol	1150	3.61 ± 0.13	-	fusel oil alcoholic whiskey fruity banana[e]
(*E*)-2-hexenal[b]	1158	1.34 ± 0.11	-	green banana aldehydic fatty cheesy[e]
4-methyl-2-heptanone	1161	-	1.66 ± 0.16	----
3-methyl-undecane	1169	0.53 ± 0.23	-	----
1-pentanol[b]	1188	0.84 ± 0.12	-	fusel oily sweet balsamic[e]
acetoin[b]	1194	2.63 ± 0.24	-	butter, cream[f]
2,2,6-trimethyl-4H-1,3-dioxin-4-one	1198	-	17.56 ± 0.68	----

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dodecane[b]	1202	2.34 ± 0.72	-	----
octanal	1237	-	4.71 ± 0.22	citrus-like, green[d]
(*Z*)-2-penten-1-ol	1246	0.93 ± 0.07	-	green phenolic nasturtium ethereal medicinal aldehydic cherry narcissus metallic fruity[e]
6-methyl-5-hepten-2-one[b]	1277	4.14 ± 0.96	-	citrus green musty lemongrass apple[e]
1-hexanol[b]	1286	3.32 ± 0.18	2.95 ± 0.12	resin, flower, green[f]
tridecane[b]	1296	0.75 ± 0.15	-	----
dimethyl trisulfide	1300	0.16 ± 0.02	-	sulfur, fish, cabbage[f]
(*Z*)-3-hexen-1-ol[b]	1307	18.60 ± 0.67	-	fresh green grassy foliage vegetable herbal oily[e]
4-methyl-2-oxo-pentanoic acid methyl ester	1333	2.32 ± 0.50	-	----
nonanal[b]	1349	1.49 ± 0.41	23.62 ± 1.55	fat, citrus, green[f]
1-heptanol[b]	1395	0.38 ± 0.15	-	musty leafy violet herbal green sweet woody peony[e]
L-leucine methyl ester	1406	-	5.64 ± 1.35	----
benzaldehyde[b]	1430	0.97 ± 0.09	-	almond, burnt sugar[f]
cis-3-hexenyl-α-methylbutyrate	1437	2.86 ± 0.15	-	fresh, green apple, sweet, fruity, pear[e]
cis-3-hexenyl valerate	1451	0.75 ± 0.04	-	green fruity apple pear kiwi banana unripe banana tropical[e]
dihydroedulan	1459	0.21 ± 0.12	-	----
linalyl acetate	1482	0.26 ± 0.02	-	sweet, fruit[f]
4,7-dimethyl-undecane	1492	-	0.24 ± 0.03	----
pentadecane[b]	1500	3.24 ± 0.26	-	waxy[e]

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benzoic acid methyl ester	1530	0.10 ± 0.01	-	phenolic wintergreen almond floral cananga[e]
benzeneacetaldehyde	1539	0.33 ± 0.03	-	honey, floral, rose, sweet, powdery, fermented, chocolate, earthy[e]
2-methyl-benzaldehyde	1545	-	0.42 ± 0.17	cherry[e]
5-methyl-1-phenyl-1-hexananone	1548	0.04 ± 0.00	-	----
(*Z*)-(*Z*)-hex-3-en-1-yl 2-methylbut-2-enoate	1608	0.29 ± 0.02	-	----
cis-muurola-4(15),5-diene	1664	0.17 ± 0.01	-	----
methyl salicylate	1667	2.96 ± 0.15	2.90 ± 0.41	wintergreen mint[e]
heptadecane[b]	1691	0.25 ± 0.03	0.18 ± 0.02	alkane[f]
α-farnesene	1708	1.78 ± 0.09	0.67 ± 0.10	wood, sweet[f]
8-heptadecene	1714	0.97 ± 0.12	-	----
benzyl alcohol[b]	1745	0.74 ± 0.08	-	sweet, flower[f]
phenylethyl alcohol	1781	0.97 ± 0.10	-	honey, spice, rose, lilac[f]
2-methyl-benzonitrile	1789	0.30 ± 0.01	-	----
methyl eugenol[b]	1888	2.46 ± 0.66	-	Clove, spice[f]
eugenol[b]	2093	1.41 ± 0.32	3.85 ± 0.40	Clove, honey[f]

[a] retention index calculated with linear alkane standard mix using the linear retention index equation. [b] Identification confirmed with authentic standard. [c] not applicable as retention index could not be calculated due to elution before the earliest alkane standard. [d]Aroma descriptor obtained from *Eur. Food Res. Techn.***2008**, *228*, 265–273. (30). [e]Aroma descriptors obtained from http://www.thegoodscentscompany.com. (16). [f]Aroma descriptors obtained from https://www.flavornet.org/flavornet.html. (21) ----- indicates that no odor descriptors could be found for this compound. – indicates that compound was not identified in the samples.

The elderflower teas evaluated herein were prepared without the addition of added ingredients that are commonly used in the preparation of elderflower syrups, tonics, and beverages. Sugar, lemon, citric acid or vinegar, and preservatives like sodium benzoate are common ingredients used in these products but can impact the headspace VOC profiles. Elderflower syrups are a cooked product, and the time and temperature at which they are processed will influence the headspace VOC profile. Elderflower syrups (made with European elderflowers, lemon, sucrose, tartaric acid, and sodium benzoate) evaluated via dynamic headspace sampling and GC/MS, show that *cis*-rose oxide, hotrienol, linalool, (*Z*)-3-hexenol, *cis*-linalool oxide (furan) and *trans*-rose oxide are some of the predominant volatiles.[128] Of these, the only compound also identified in the present study is (*Z*)-3-hexenol, which was present at 3.51 ± 0.41% in fresh flowers and 18.60 ± 0.67% in dry flowers. Heptanal and nonanal were also identified in the syrup, and levels ranged from 15.5 to 80.7 ng mL^{-1} and 13.1 to 33.2 ng mL^{-1}, respectively.[35] These compounds represent < 1-3% of the total concentration of VOCs and are significantly lower as compared with the levels identified in the present study. In another study of elderflower syrup with European elderflowers, a variety of process parameters, such as extraction temperature, time, and syrup composition, were evaluated for their impacts on VOCs profiles.[81] While the concentration of volatile compounds was dependent upon how the syrup was made, (*Z*)-rose oxide, linalool, (*E*)-3-hexen-1-ol, (*E*)-rose oxide, 1,1,6-trimethyl,1,2-dihydronaphthalene, (*Z*)-linalool oxide, and 2- and 3-methyl-1-butanol were some of the most concentrated compounds identified. In comparison to our results, (*Z*)-3-hexen-1-ol was identified to be the isomer present, and 3-methyl-1-butanol was present in dry ground flowers but only at 3.61 ± 0.13%. Not surprisingly, the volatile profiles from European elderflower syrups do not correspond well with the blue elderflower teas. However, future studies evaluating syrup made from blue elderflowers, could provide better insight into how the aroma,

flavor and biological potential differs from syrups made from European subspecies. Studies could also include the American elderflower, which has not been evaluated for its headspace VOC profile before and results could further differentiate these subspecies.

The results from this study present the first information on the phenolic and VOC composition of the blue elderflower, the subspecies native to the western region of North America. The phenolic profile elucidated unique characteristics of this subspecies compared to the other more widely used subspecies, *S. nigra* ssp. *nigra* and *S. nigra* ssp. *canadensis,* namely that IR was the predominant phenolic compound measured. A novel phenolic compound, 5-hydroxypyrogallol hexoside, was also identified in the blue elderflowers. Furthermore, the headspace VOC profile of the fresh and dry flowers as well as teas made from both types of flowers showed distinctive aroma profiles, highlighting that how elderflowers are processed post-harvest will impact the volatile compounds present and their relative concentration. Methyl eugenol and 5-HPG hexoside were identified for the first time in elderflowers. Further sensory evaluation would help determine if consumers differentiate between products using various subspecies of elderflower.

Acronyms

5-HPG, 5-hydroxypyrogallol; DAD, diode array detection; DHF, dry homogenized flowers; DWF, dry whole flowers; FHF, fresh homogenized flowers; FLD, fluorescence detection; FWF, fresh whole flowers; GC/MS, gas chromatography mass spectrometry; HCl, hydrochloric acid; HPLC, high performance liquid chromatography; HS-SPME, headspace solid phase microextraction; IR, isorhamnetin-3-*O*-rutinoside; LLOD, lower limit of detection; LLOQ, lower limit of quantitation; QTOF-MS/MS, quadrupole time of flight tandem mass spectrometry.

Author contributions

K.R.U.: methodology, conducting analyses, writing the first manuscript draft, and editing.

A.E.M.: project conceptualization, funding, administration, experimental design, supervision, and writing, editing and reviewing the manuscript.

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Conflict of interest

The authors declare no competing financial interest.

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Chapter 2. Supporting Information

Table S1. Base peak ion *m/z* and average match factor rating for volatile compounds identified in fresh elderflowers and tea made with fresh elderflowers

Compound	Base peak *m/z*	Avg match factor (%)
2,4-dimethyl-1-heptene	43	97.2
n-butyl ether	57	88.1
hexanal	44	97.9
o-formic acid, triisobutyl ester	57	88.5
heptanal	41	99.1
4-methyl-2-heptanone	43	90.3
D-limonene	68	97.0
2,2,6-trimethyl-4H-1,3-dioxin-4-one	43	84.5
hexyl acetate	43	94.9
octanal	41	94.8
cis-3-hexenyl acetate	43	92.8
1-hexanol	56	93.3
(*Z*)-3-hexen-1-ol	41	98.4
4-methyl-2-oxo-pentanoic acid methyl ester	57	95.1
2-methyl-propanoic acid 3-hexenyl ester	67	93.4
nonanal	41	97.7
butanoic acid hexyl ester	70	87.8
L-leucine methyl ester	86	89.8
(*Z*)-butanoic acid-3-hexenyl ester	67	92.5
cis-3-hexenyl-α-methylbutyrate	67	95.8
4,7-dimethyl-undecane	57	91.5
pentadecane	57	98.5
1-pentadecene	55	93.5
2-methyl-benzaldehyde	91	90.2
(*Z*)-hexanoic acid 3-hexenyl ester	82	94.1
germacrene D	161	92.1
methyl salicylate	120	97.1
α-farnesene isomer 1	93	85.6
heptadecane	57	83.9
α-farnesene isomer 2	93	97.6
8-heptadecene	55	97.3
6,9-heptadecadiene	67	85.7
methyl eugenol	178	98.2
eugenol	164	98.3
(*E*)-1,2-dimethoxy-4-(1-propenyl)-benzene	178	87.6
1,2,3-trimethoxy-5-(2-propenyl)-benzene	208	91.8

Table S2. Base peak ion *m/z* and average match factor rating for volatile compounds identified in dry elderflowers and tea made with dry elderflowers

Compound	Base peak *m/z*	Avg match factor (%)
isocyanato-methane	57	83.2
2,4-Dimethyl-1-heptene	43	97.2
3-methyl-butanal	41	90.4
pentanal	44	95.1
n-butyl ether	57	88.1
3-methyl-nonane	57	86.8
methyl isovalerate	74	88.9
decane	43	95.7
hexanal	44	97.9
2-nitro-propane	43	87.6
1-penten-3-ol	57	92.3
o-formic acid, triisobutyl ester	57	88.5
heptanal	41	99.1
3-methyl-1-butanol	55	97.1
(*E*)-2-hexenal	41	98.1
4-methyl-2-heptanone	43	90.2
3-methyl-undecane	57	89.2
1-pentanol	42	93.7
acetoin	45	94.1
2,2,6-trimethyl-4H-1,3-dioxin-4-one	43	84.5
dodecane	57	97.1
octanal	41	94.8
(*Z*)-2-penten-1-ol	57	88.1
6-methyl-5-hepten-2-one	43	97.2
1-hexanol	56	93.3
tridecane	57	95.4
dimethyl trisulfide	126	88.3
(*Z*)-3-hexen-1-ol	41	98.4
4-methyl-2-oxo-pentanoic acid methyl ester	57	95.1
nonanal	41	97.7
1-heptanol	70	89.9
L-leucine methyl ester	86	89.8
benzaldehyde	77	97.5
cis-3-hexenyl-α-methylbutyrate	67	95.8
cis-3-hexenyl valerate	67	96.1
dihydroedulan	179	82.1
linalyl acetate	71	84.7
4,7-dimethyl-undecane	57	91.5
pentadecane	55	98.5
benzoic acid methyl ester	105	84.9
benzeneacetaldehyde	91	85.1
2-methyl-benzaldehyde		

5-methyl-1-phenyl-1-hexananone	105	80.4
(*Z*)-(*Z*)-hex-3-en-1-yl 2-methylbut-2-enoate	55	94.9
cis-muurola-4(15),5-diene	161	82.3
methyl salicylate	120	97.1
heptadecane	57	83.9
α-farnesene	93	97.6
8-heptadecene	55	97.3
benzyl alcohol	79	93.2
phenylethyl alcohol	91	91.2
2-methyl-benzonitrile	117	93.3
methyl eugenol	178	98.2
eugenol	164	98.3

Chapter 3. Characterization of cyanogenic glycosides and phenolic compounds in blue elderberry juice (*Sambucus nigra* ssp. *cerulea*) during thermal processing

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Abstract

Blue elderberry (*Sambucus nigra* ssp. *cerulea*) is a plant native to the western region of North American and could be used in similar food applications and supplements as the more commonly used European black elderberry (*S. nigra* ssp. *nigra*). Elderberries contain both health promoting phenolic compounds as well as potential toxic cyanogenic glycosides thus they should be processed carefully to create safe products for consumers. Herein, cyanogenic glycosides were characterized for the first time in the juice of the blue elderberry, and the thermal stability of the cyanogenic glycosides as well as the main phenolic compounds were evaluated through juice processing. Cyanogenic glycosides concentrations in raw juice included neoamygdalin (430.0 ± 15.8 ng L^{-1}), sambunigrin (264.5 ± 14.6 ng L^{-1}), and prunasin (42.8 ± 6.5 ng L^{-1}), levels lower than other elderberry subspecies. Elderberry juice was cooked at 72 and 95 °C for two hours, and the phenolic compounds and cyanogenic glycosides were quantified in juice over time using HPLC-DAD and UHPLC-QQQ-MS/MS, respectively. Anthocyanins were the most heat-labile phenolic compounds, degrading 30% at 72 °C and 60% at 95 °C, after two hours. The main flavonols, rutin and isorhamnetin 3-glucoside, were more thermally stable, retaining more than 95% of their original concentration even after 2 h at 95 °C. Cyanogenic glycosides degraded significantly after 2 h of thermal processing, except for prunasin at 72 °C, which did not decrease significantly. Measuring the cyanogenic glycosides and degradation of phenolic compounds in blue elderberry juice can better inform processors using this native fruit to make safe products for consumers while maintaining bioactive compounds.

Keywords: bioactive, heat treatment, thermal stability, fruit juice, HPLC, anthocyanins

Introduction

Elderberry (*Sambucus nigra* L.) is a small fruit commonly used in food, beverages, and in supplements. Most products available use the European subspecies (*S. nigra* ssp. *nigra*) which has established cultivars and growing regions, and for which there is knowledge on composition. The blue elderberry (*S. nigra* ssp. *cerulea*) is native to the western regions of North America, grows in a wide range of climates and soil types, and is notable due to a white bloom caused by yeast that make the dark purple berries appear light blue.[11] In California, in addition to growing wild, typically in riparian environments, blue elderberry is grown in hedgerows to improve biodiversity near other crops.[17,111] Unlike the commercialized European elderberry subspecies, the blue elderberry has not yet been bred for specific cultivars/genotypes and is not used in commercial production of elderberry products. Due to the environmental benefits of the blue elderberry, like improved water, soil, and air quality in addition to food and shelter for pollinators, there is rising interest in utilizing the fruit for commercial applications; especially as a juice, in beverages and supplements. At the same time, elderberry-based products have increased in popularity as consumers look for natural ways to support their immune system.[9] A better understanding of how thermal processing influences key chemical components of this fruit may help to increase the use of this native and sustainable plant in value-added products in the US.

Numerous important crop plants contain cyanogenic glycosides including cassava, sorghum, lima beans, the pits of *Prunus* species (e.g., almonds, cherries, apricot) as well as elderberry.[6,130] Cyanogenic glycosides are nitrile-containing plant secondary defense compounds that can present a potential toxicity risk to consumers yet cyanogenic glycosides themselves are not toxic.[130] However, they can release hydrogen cyanide (HCN) when the plant material is either damaged, chewed or digested through the action of endogenous β-glucosidase and/or gut

microorganisms. Hydrolysis results in the production of sugar(s) and a cyanohydrin that spontaneously decomposes to HCN and a ketone or aldehyde .[130] Exposure to HCN can lead to rapid pulse, headache, vomiting, diarrhea, convulsions, and in extreme cases, death.[130] Chronic consumption of CNGs, particularly in regions that rely on a cyanogenic staple food but have other nutritional inadequacies, can also cause complications and may lead to diseases such as konzo or tropical ataxic neuropathy.[130] While individual metabolism of CNGs can vary significantly and is impacted by the structure of the CNG, the acute lethal oral dose of cyanide in humans is 0.5 to 3.5 mg/kg body weight.[131]

To make foods safe, cyanogenic glycosides are frequently hydrolyzed through processes including grinding, pounding, boiling, steaming or during soaking or fermentation in water.[132] Thermal treatments have also been used to effectively decrease levels of cyanogenic glycosides in foods.[1,133,134] Commercial elderberry juice is thermally processed to inactivate enzymes, improve microbiological stability, and to degrade the potentially toxic cyanogenic glycosides (CNGs)[135] found in this berry which include amygdalin, dhurrin, linamarin, and sambunigrin (Figure 1).

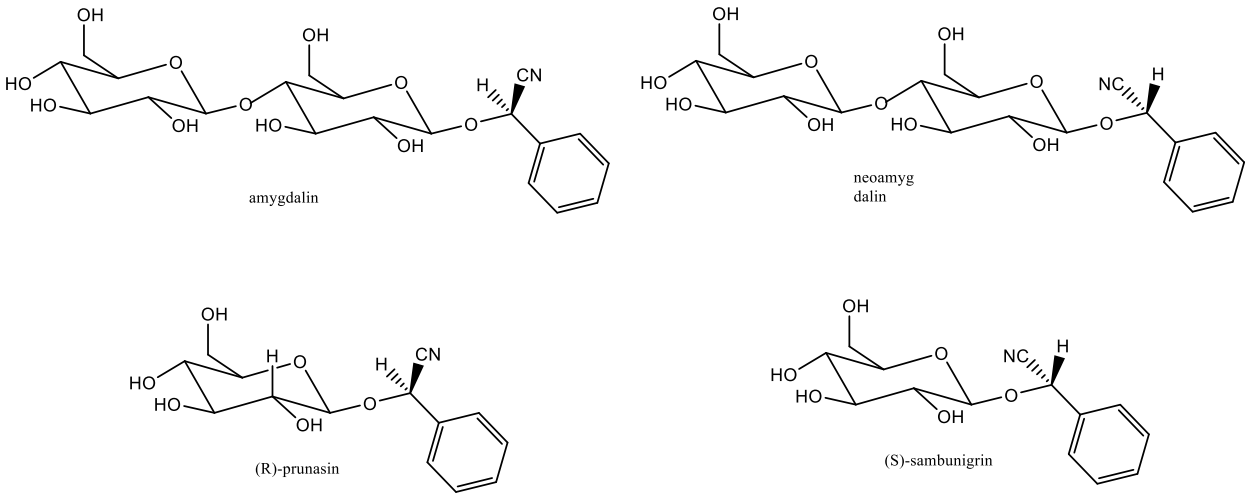

Figure 1. Cyanogenic glycosides identified in elderberry

The range and content of cyanogenic glycosides in the blue elderberry have not yet been identified. However, there was an occurrence of people getting sick after consuming raw blue elderberry juice at a at a religious gathering in Monterey County, California in 1983.[136] Therefore understanding levels of cyanogenic glycosides in this subspecies is critical if it is going to be considered for use in commercial products. In European elderberry, sambunigrin is the main CNG in all parts of the plant, with the highest levels in the leaves (28.82 ± 1.10 to 153.31 ± 3.26 μg g^{-1}) and the lowest levels in the ripe berries (0.08 ± 0.01 to 0.77 ± 0.08 μg g^{-1}).[6] Sambunigrin is a diastereoisomer of prunasin, containing L-glucose instead of D-glucose.[9] In the American (*S. nigra* ssp. *canadensis*) subspecies, amygdalin, dhurrin, linamarin, and sambunigrin were identified in the skin, seed, and juice of the berry as well as in the stem.[60] Juice of the American elderberry contained the lowest levels of CNGs relative to the skin, seeds and stems, averaging about 4 μg g^{-1}, while the stems had the highest concentrations (8 to 10 μg g^{-1}) in the two cultivars evaluated.[60]

A recent study demonstrated that the total phenolic content of the blue elderberry is similar to the European and American subspecies, however this subspecies has significantly lower levels of anthocyanins.[39] The dominant phenolic compounds identified in the blue elderberry include the flavonols rutin and isorhamnetin glucoside, the phenolic acid chlorogenic acid, a novel phenolic compound tentatively identified as 5-hydroxypyrogallol hexoside (5-HPG), and the anthocyanins cyanidin 3-glucoside (cyn 3-glu) and cyanidin 3-sambubioside (cyn 3-sam).[39]Anthocyanins are responsible for the brilliant blue-purple color of elderberries. At the same time, anthocyanidins are desired by consumers for their potential health promoting bioactivity.[137] Unfortunately, heat processing and pasteurization can lead to the degradation of anthocyanins resulting in a loss in color or a formation of brown polymers, possibly impacting the acceptability of the final product.

Elderberry juice and extracts have been evaluated for their thermal stability, which have shown that anthocyanins degrade following first-order reaction kinetics.[138] The stability of anthocyanidins can be reinforced via intra- and inter-molecular interactions with protective structures and flavonoids through a phenomenon termed copigmentation.[139] Notably, acylated anthocyanins, such as those found in American elderberry like cyanidin 3-coumaroyl-sambubioside, are sometimes more stable during thermal processing due to protective properties of the coumaroyl group folding over the flavylium ion.[139] Like the European elderberry, the blue elderberry does not contain acylated anthocyanins.[3912] The thermal stability of anthocyanidins in blue elderberry juice has not yet been evaluated. However, as elderberry juice and extracts are frequently thermally processed to make products, such as jam, syrup, or gummies, it is important to understand the thermal degradation of anthocyanidins in the juice from blue elderberry.

The purpose of this study was to determine the stability of the cyanogenic glycosides and main phenolic compounds (i.e. rutin, isorhamnetin 3-glucoside, chlorogenic acid, cyn 3-glu and cyn 3-sam) in blue elderberry juice cooked at 72 °C and 95°C for two hours to elucidate the kinetics of degradation of these important compounds.

Materials and methods

Chemicals

HPLC-grade methanol (MeOH), and LCMS grade acetonitrile, methanol, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Ultrapure water (18.2 MΩ) was obtained from a Milli-Q water system (Millipore Sigma, Burlington, MA). Prunasin was also obtained from Millipore Sigma. HPLC-grade acetonitrile (ACN), rutin (quercetin 3-*O*-rutinoside), isorhamnetin 3-*O*-glucoside, caffeic acid, chlorogenic acid, (+)-catechin, protocatechuic acid, ammonium

formate, and amygdalin were purchased from Sigma-Aldrich (St. Louis, MO). Cyanidin 3-*O*-sambubioside chloride (cyn 3-sam) and cyanidin 3-*O*-glucoside (cyn 3-glu) were purchased from ExtraSynthese (Genay Cedex, France).

Plant material

Ripe berries were harvested in July 2019 from a farm in Winters, CA at latitude and longitude coordinates of 38.634884, -122.007502. Fruit was selected from all sides of the plant at a variety of heights to obtain a representative sample of berries from each plant. Only fully ripe berries (i.e., no green berries present in the cyme, but instead dark blue-purple skin). About 5 kg were harvested from each of shrub in three hedgerows. The plant material was transported to University of California, Davis on ice in plastic gallon bags within two hours of harvest and stored at -20 °C until analysis.

Preparation of elderberry juice

Elderberry juice was prepared from 300 g previously frozen berries, thawed at room temperature for one hour in a mesh bag in a metal bowl. Thawed berries were juiced with a manual fruit press (EJWOX, Santa Ana, CA). The juice obtained was then aliquoted into glass vials and sealed with a screw cap to avoid evaporation (2 mL juice in each). A time-zero aliquot of juice was immediately placed on ice for analysis. An aliquot was also analyzed for Brix (Palette digital refractometer, Atago, Bellevue, WA) and pH measurements (SevenMulti pH meter, Mettler Toledo, Columbus, OH).

Thermal processing of elderberry juice

A hot water bath was prepared using an immersion circulator (InstantPot AccuSlim, Downers Grove IL), set to the desired cooking temperature (either 72.0 or 95.0 °C, which reflect a conventional temperature used for juice thermal processing and a more intensive heat treatment, respectively). The temperature was also monitored with a thermometer in the water bath. Vials of juice randomized and place into the hot water bath and the bath was covered during cooking. Duplicate vials were removed at the following times: 15, 30, 45, 60, 75, 90, 105, and 120 min. An extended processing time was used to observe degradation of more heat-stable compounds as well as to make comparisons to other studies that processed juice for multiple hours at these temperatures. Once removed from the hot water bath, vials were placed immediately into an ice bath for 15 min. Then from each vial, 1 mL of juice was placed in a microcentrifuge tube and centrifuged at 4 °C, 15,000 rpm (21,130 rcf) for 15 min (Eppendorf, Hamburg, Germany). Next, the supernatant was diluted 1:10 with 1% formic acid in water, filtered with 0.2 µm PTFE filter, and placed in an HPLC vial for analysis. Five replicate juice samples were prepared and cooked at both temperatures.

Analysis of phenolic compounds via HPLC-DAD

Phenolic compounds were analyzed via an Agilent 1200 HPLC equipped with a binary pump, autosampler, temperature-controlled column compartment, and diode array detector (DAD). A 20 µL aliquot of diluted juice was injected into the system and onto an Agilent Poroshell ZORBAX SB-Aq column (2.1 x 100 mm, 3.5-micron particle size) at 35 °C. The mobile phases were: (A) 1% formic acid in water; and (B) acetonitrile, set to 1.00 ml min^{-1}. The gradient was as follows: 0 min, 5% B, 4 min 15% B, 6 min 30% B, 6.5 – 8.5 min 95% B, 9 – 11 min 5% B. Compounds were detected at 280 nm (hydroxybenzoic acids and flavan-3-ols), 320 nm

(hydroxycinnamic acids), 360 nm (flavonols), and 520 nm (anthocyanins). Compounds were identified by comparing retention time and absorbance spectra to those of standards. Peak areas were used for relative quantitation.

Extraction of cyanogenic glycosides

Composite juice samples were prepared by combing equal aliquots of the time points 0, 15, 30, 60, and 120 minutes of thermal processing for both temperatures for each juice prepared. To extract CNGs for blue elderberry juice, 0.500 mL of juice was mixed with 2.00 mL of methanol in a 5 mL centrifuge tube, then sonicated at 30 °C for 30 min. After sonication, 1.00 mL of extract was transferred to a 1 mL microcentrifuge tube and centrifuged at 15,000 rpm (21,130 rcf) at 4 °C for 15 min. The supernatant was collected and filtered through 0.22 µm PTFE into an HPLC vial and used for analysis. Five replicate extractions were made of raw elderberry juice (time 0) and triplicate extractions were made for the other time/temperature juices.

Analysis of cyanogenic glycosides via UHPLC-QQQ-MS/MS

CNGs were analyzed via ultra-high performance liquid chromatography with electrospray ionization and triple quadrupole tandem mass spectrometry (UHPLC-QQQ-MS/MS) using an Agilent 1290 Infinity HPLC and 6460 mass spectrometer (Santa Clara, CA). The UHPLC was equipped with a binary pump with an integrated vacuum degasser (G4220A), an autosampler (G4226A) with thermostat (G1330B), and a thermostated column compartment (G1316C). CNGs were separated using a Kinetex F5 column (2.1 × 150 mm, 2.6 µm, Phenomenex, Torrance, CA) at 40.0 °C. The mobile phase consisted of a linear gradient of 1 mM ammonium formate in water (A) and 1 mM ammonium formate in 650:50 MeOH:ACNe (B) as follows: 5% B, 0–5 min; 100%

B 5-5.50 min, 95% A 5.60-6.50 min. The flow rate was 0.400 mL/min, and the injection volume was 5.0 μL. The CNGs were analyzed using negative ESI mode. The drying gas temperature was 300 °C and the flow rate was 8.0 L min^{-1}. The sheath gas temperature and flow rate were 350 °C and 11.0 L min^{-1}, respectively. The nebulizer gas pressure and capillary voltage were 45 psi and 3.5 kV, respectively. The fragmentor voltage was 160 V for amygdalin and 100 V for sambunigrin. The dwell time was 100 ms for amygdalin and 200 ms for sambunigrin. The collision energy was set to 12 V for amygdalin, 0 V for sambunigrin. The multiple reaction monitoring (MRM) mode was utilized to analyze amygdalin and sambunigrin. Quantification of CNGs was performed using external calibration curves using standard addition at levels of 500, 100, 50, and 5 ng L^{-1}. For amygdalin, the area of *m/z* 456.2 (precursor ion) to *m/z* 323.1 (product ion) was measured. For sambunigrin, the area of *m/z* 340.3 (precursor ion) to *m/z* 294.2 (product ion) was measured.

Data handling

Half-life values of phenolic compounds were calculated by plotting the natural log of C_0/C ratio vs heating time *t*, where C_0 is the initial concentration of a compound, C is the concentration of the same compound at time *t* in hours. The slope calculated from the figure is k (rate constant). Longitudinal Analysis of variance (ANOVA) was performed with Tukey's post-hoc test with *p* value at 0.05. Excel used for average and standard deviation and R Studio was used for ANOVA and post hoc analysis (R Core Team, Boston, MA).

CNG data was analyzed using MassHunter Quantitative Analysis (Agilent, Santa Clara, CA) to obtain peak areas for the targeted compounds. Microsoft Excel was used to create the calibration curves and determine CNG concentrations in samples (Redmond, WA).

Results and discussion

Analysis of cyanogenic glycosides

The concentrations of cyanogenic glycosides (CNGs) were quantified in raw and cooked blue elderberry juice for the first time. Results indicate that neoamygdalin (the S-epimer of amygdalin), sambunigrin and prunasin are the primary CNGs in blue elderberry (Table 1). Concentration of neoamygdalin (430.0 ± 15.8 ng L^{-1}) were significantly higher than sambunigrin (264.5 ± 14.6 ng L^{-1}) and prunasin (42.8 ± 6.5 ng L^{-1}). Neoamygdalin has been measured in raw bitters almonds in concentrations lower than amygdalin.[133] In studies of American and European elderberry, sambunigrin is typically major CNG identified. Levels of total CNGs in blue elderberry are lower than American and European elderberry. European elderberry CNG levels range from 0.08 ± 0.01 to 0.77 ± 0.08 μg g^{-1} depending on the elevation and growing location.[6] CNG levels in American elderberry juice range from 0.29 to 2.36 μg mL^{-1}. Differences between the subspecies may be due to genetic variation, impact of growing environment such as altitude, or methodology used to extract and analyze CNG content in the fruit and fruit juice, including how berries were handled prior to juice (frozen or used fresh) and juicing method.

Table 1. CNG levels in raw and cooked blue elderberry juice (ng L^{-1})

Cyanogenic glycoside	Time processed (min)	Cooked at 72 °C	Cooked at 95 °C
Neoamygdalin	0	430.0 ± 15.8 b	430.0 ± 15.8 d
	15	284.0 ± 59.8 a	375.5 ± 23.4 c
	30	407.7 ± 24.8 b	255.9 ± 37.9 b
	60	324.9 ± 20.2 a	254.0 ± 22.4 b
	120	316.9 ± 31.3 a	152.0 ± 56.2 a
Sambunigrin	0	264.5 ± 14.6 c	264.5 ± 14.6 b
	15	207.8 ± 9.3 a	206.9 ± 8.7 ab
	30	211.6 ± 11.2 a	238.8 ± 36.8 a
	60	191.5 ± 13.6 a	220.2 ± 9.0 a
	120	229.1 ± 5.3 b	188.6 ± 39.4 a
Prunasin	0	42.8 ± 6.5 a	42.8 ± 6.5 b
	15	32.1 ± 4.0 a	25.3 ± 8.1 a
	30	31.9 ± 2.4 a	29.1 ± 4.5 a
	60	29.8 ± 4.3 a	33.9 ± 11.6 ab
	120	29.7 ± 9.5 a	21.0 ± 7.1 a
Total	0	737.4	737.4
	15	524.0	607.7
	30	651.2	523.9
	60	546.2	508.1
	120	575.6	361.5

Concentrations of a compound in a column (cooking temperature) with different letters indicate a significant difference ($p < 0.05$).

The degradation of neoamygdalin > sambunigrin > prunasin was observed during cooking and the rate of degradation was faster at 95 °C as compared to 72 °C (Table 1). However, degradation in juice at 72 °C was not linear, such that sambunigrin levels in juice cooked at significantly increased during the final timepoint measured (229.1 ± 5.3 ng L^{-1} at 120 min compared to 191.5 ± 13.6 ng L^{-1} at 60 min). This may be attributed to neoamygdalin breaking down resulting in sambunigrin and a glucose molecule, an equivalent pathway to amygdalin degrading to prunasin and a glucose molecule. However, some of the resulting sambunigrin from that reaction would also have to be degrading since the decrease in concentration of neoamygdalin

did not cause an equivalent increase in sambunigrin. An increase in sambunigrin at the end of the processing time was not observed in the juice cooked at 95 °C. In the juice processed at 95 °C, the combined concentration of neoamygdalin and sambunigrin decreased at each measured time point and did not increase at any time points like the juice processed at 72 °C.

Prunasin levels did not significantly change in the elderberry juice cooked at 72 °C (42.8 ± 6.5 ng L^{-1} in raw juice and 29.7 ± 9.5 ng L^{-1} at 120 min) but prunasin did degrade significantly when processed at 95 °C (42.8 ± 6.5 ng L^{-1} in raw juice to 21.0 ± 7.1 ng L^{-1} at 120 min). It appears that sambunigrin is more stable than prunasin in the elderberry juice; retaining about 70% of the original concentration in the juice heated to 95 °C. Neoamygdalin levels decreased significantly in the elderberry juice at both processing temperatures, with increased degradation at 95 °C as compared with the treatments at 72 °C. As previously mentioned, sambunigrin is the expected breakdown product from neoamygladin, but sambunigrin levels did not have concomitant increase due to thermal degradation of sambunigrin as well. Thermal processing has been seen to degrade CNGs in elderberry, flaxseed, and almond in previous studies.[1,133,134] Furthermore, studies have seen enzymatic activity contributing to the breakdown of CNGs in nuts to reduce after exposure to heat.[133,140] If the β-glucosidases for the CNGs present in blue elderberry are similar, they would also be inactivated during thermal processing at 72 and 95 °C, indicating thermal degradation is the main contribution to CNG levels decreasing in the present study. Because enzymatic degradation of CNGs was not measured during the thawing and juicing steps, the impact of the enzymes before the thermal processing cannot be evaluated here.

The presence of neoamygdalin instead of amygdalin (which was present in trace amounts below the LOQ) is unexpected. Amygdalin can convert to neoamygdalin with heat and in alkaline conditions. However, herein the raw (i.e., non-thermally processed) elderberry juice had

significantly higher levels of neoamygdalin as compared to amygdalin. In a study of amygdalin content in almond varieties, amygdalin was found to convert to neoamygdalin during extraction (~35% of the standard in methanol/water 40:60 v/v), but the addition of acetic acid prevented the conversion.[141] Blue elderberry naturally contain citric and malic acids, with an average titratable acidity of 0.60 ± 0.10 to 0.65 ± 0.07 g citric acid per 100 g FW.[39] The average pH value of the juices in the present study was 3.76 ± 0.11. Therefore, there may not be enough acid in the matrix to prevent the conversion. In contrast, another study of amygdalin and derivatives in almonds found that heat of cooking caused neoamygdalin and amygdalin amide to convert to amygdalin, which was not observed in the present study.[133] Further analysis of conversion of amygdalin to neoamygdalin in the blue elderberry could uncover why this epimer is dominant.

The total levels of CNGs measured here are much lower than CNG concentrations found in European or American elderberry. In a study of European elderberries evaluated at various growing locations and altitudes found that sambunigrin levels range from 0.08 ± 0.01 to 0.77 ± 0.08 μg g^{-1}.[6] A nearly 10-fold difference in concentrations between elderberry samples highlights the variation on CNG levels due to differences in growing conditions and environmental factors like sun exposure and temperature fluctuations. Furthermore, evidence of CNGs degrading with thermal processing has been evaluated in European elderberry products: when sambunigrin levels were measured in raw and cooked elderberry juice and other products, heating of elderberry juice (prepared by pressing fresh berries in a plastic bag) reduced the level of sambunigrin, from 18.8 ± 4.3 mg kg^{-1} to 10.6 ± 0.7 mg kg^{-1}.[1] Liqueur, tea, and spread (0.8 ± 0.21, 0.38 ± 1.7, 3.8 ± 0.8 mg kg^{-1}) also had significantly lower CNG concentrations as compared to the raw and cooked juice.

American elderberry was evaluated for concentration of CNGs in the seeds, juice, skin, and stem of two genotypes: Ozone and Ozark.[60] Elderberry juice was prepared by thawing

previously frozen berries in a plastic bag and gently pressing to release juice. The juice of these elderberries contained amygdalin, dhurrin, prunasin/sambunigrin (isomers not separated), and linamarin. Total concentrations of these four CNGs was 4.01 μg g^{-1} in Ozone and 3.66 μg g^{-1} in Ozark elderberries. The levels of amygdalin and prunasin/sambunigrin were almost equal in Ozone (1.57 and 1.45 μg g^{-1} respectively) but in Ozark, prunasin/sambunigrin levels were much higher than amygdalin (2.36 and 0.36 μg g^{-1} respectively). These concentrations are much higher than the levels found in the present study, as raw blue elderberry juice had a total CNG concentration of only 0.737 μg g^{-1}. Because CNGs are formed from phenylalanine, it is possible that the blue elderberry had limited stock of this key material to create CNGs. An alternative reason may be that blue elderberry may have less expression of the genes needed to form CNGs like sweet almonds compared to bitter almonds.[142] CNGs may have also been degraded during juice preparation due to native β-glucosidases. A future study should investigate the impact of freeze-thaw cycles on the activity of β-glucosidase in elderberries because elderberries are frequently frozen before processing because they can spoil quickly if only refrigerated.

Thermal stability of phenolic compounds

Two cooking temperatures were investigated to understand the impact of temperature on the degradation rates of the phenolic compounds in blue elderberry juice. The pH and soluble solids were evaluated for the five juice replicates to ensure the juices were similar for the cooking process. The average pH value of the juices was 3.76 ± 0.11 and the average Brix reading was 16.2 ± 1.1%. The major phenolic compounds in elderberry juice were measured via HPLC-DAD and include 5-hydroxyprogallol hexoside (5-HPG), which is a novel phenolic compound tentatively identified for the first time by Uhl et al. 2022[39] chlorogenic acid, rutin, isorhamnetin-3-*O*-

glucoside, cyn 3-sam, and cyn 3-glu. Whereas levels of cyn 3-sam and cyn 3-glu decreased to 82.2 ± 6.9 % and 79.3 ± 6.3 %, respectively (Figure 2), more than 98% of the original concentration of 5-HPG, rutin, isorhamnetin-3-*O*-glucoside (Figure 3) and chlorogenic acid (Figure 4) remained after two hours. At the higher cooking temperature (95 °C), the anthocyanins again experienced significant degradation, retaining only 33.2 ± 4.6 % (cyn 3-sam) and 36.8 ± 5.5 % (cyn 3-glu) of the original concentration after cooking two hours (Figure 5). In a separate study of the thermal stability of elderberry juice, 15% of cyn 3-sam and cyn 3-glu were retained in juice as compared to control juice.[1] Szalóki-Dorkó, et al. (2016) demonstrated that the more complexly glycosylated anthocyanins cyn 3-sam is more stable during thermal process as compared to cyn 3-glu.[143] The results of our study are similar to Oancea et al. (2018) which showed after 90 min at 100 °C, total anthocyanin content degraded 58 %.[58] However, that study also observed an increase in total phenolic (increase of 8 %) and total flavonoid content (increase of 24 %) after 60 min, followed by a gradual decrease, which was not observed herein. If sample vials were sealed well to protect from any loss of moisture, this increase in concentrations may be due to the release of phenolic compounds bound to the cell well or other polysaccharides, which can be released with the assistance of pectinase treatments.[135,143]

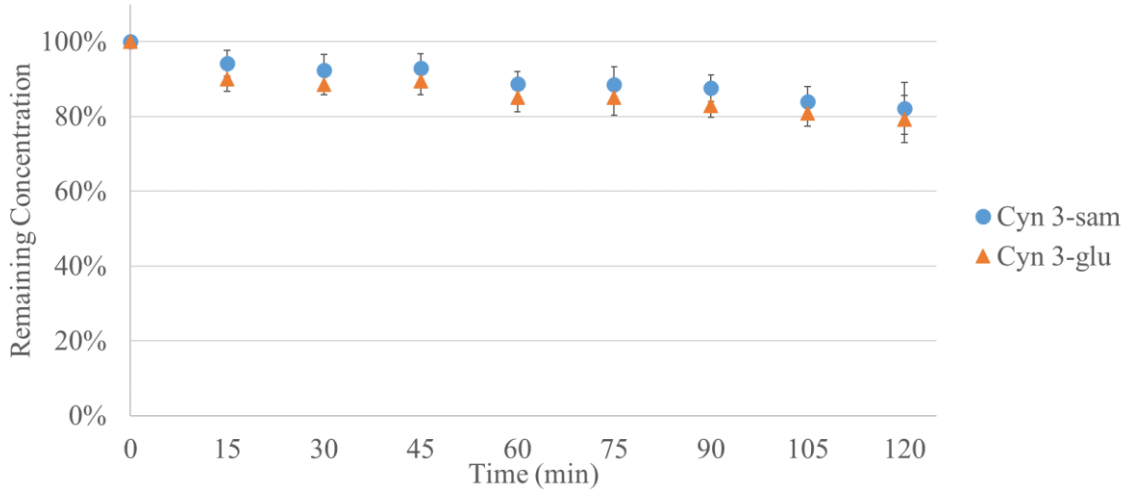

Figure 2. Concentration of anthocyanins in blue elderberry juice cooked at 72 °C for two hours

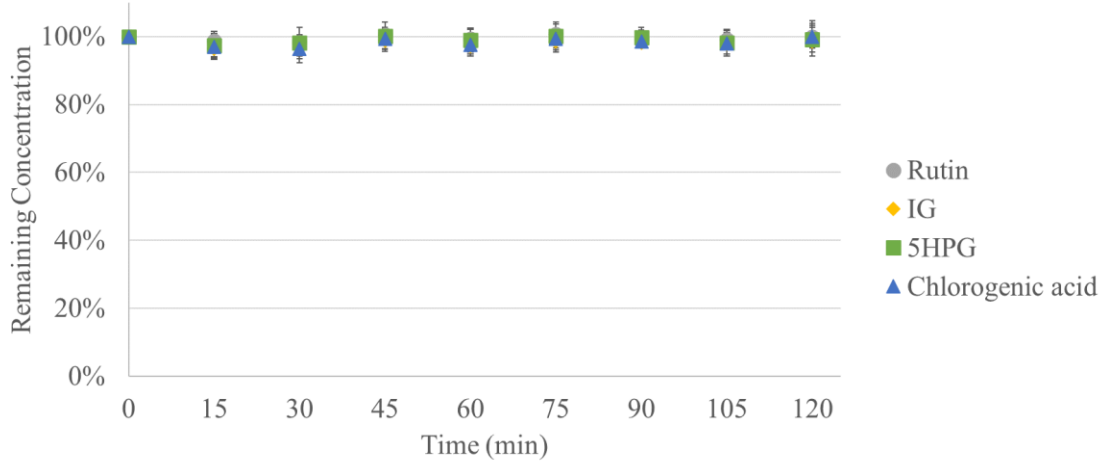

Figure 3. Concentration of rutin, isorhamnetin 3-glucoside (IG), 5-hydroxypyrogallol hexoside (5-HPG), and chlorogenic acid in blue elderberry juice cooked at 72 °C for two hours

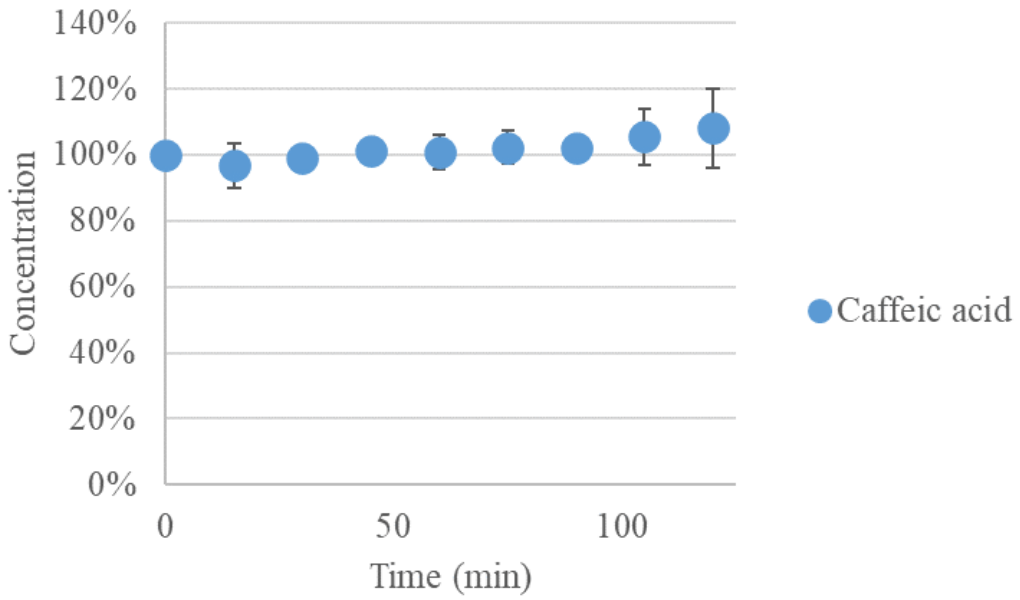

Figure 4. Concentration of caffeic acid in blue elderberry juice cooked at 72 °C for two hours

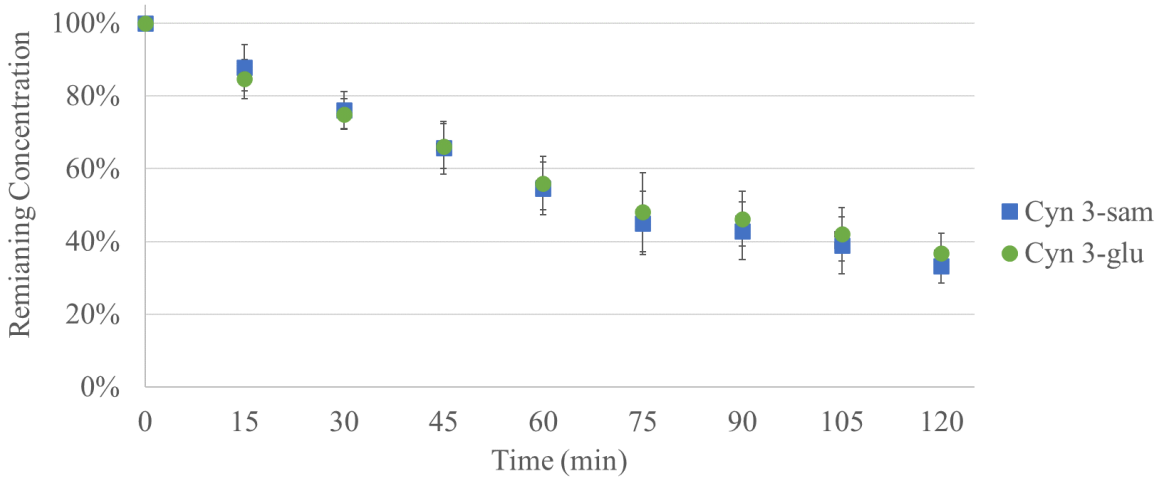

Figure 5. Concentration of anthocyanins in blue elderberry juice cooked at 95 °C for two hours

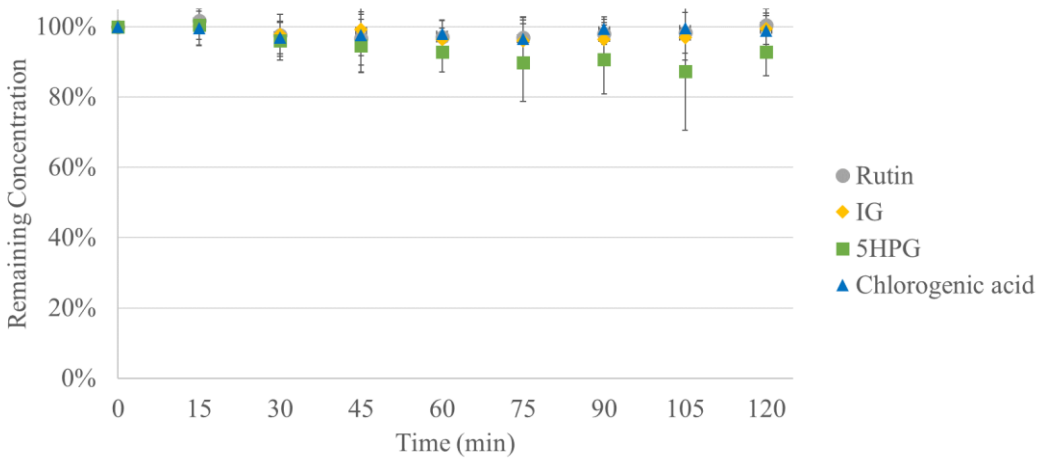

Figure 6. Concentration of rutin, isorhamnetin 3-glucoside (IG), 5-hydroxypyrogallol hexoside (5-HPG), and chlorogenic acid in blue elderberry juice cooked at 95 °C for two hours

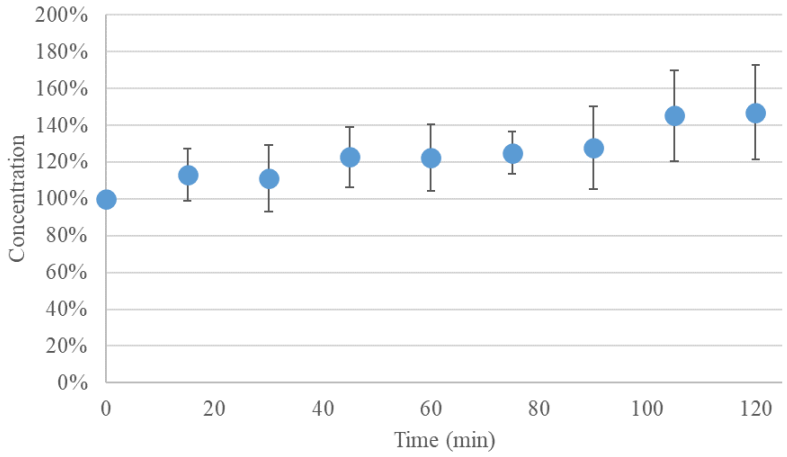

Figure 7. Concentration of caffeic acid in blue elderberry juice cooked at 72 °C for two hours

The rate of anthocyanin degradation followed first-order reaction kinetics at both experimental temperatures. The calculated rate constants *k* and half-life values are presented in Table 2. At 72 °C, cyn 3-sam is slightly more stable with a half-life of 6.80 h, whereas cyn 3-glu has a shelf-life of 5.25 h. This trend was reversed at 95 °C, where cyn 3-glu had a slightly longer half-life at 1.33 h while cyn 3-sam was only 1.23 h. [20] Cyn 3-sam in elderberry juice cooked at 95 °C at pH 1 had a half-life value of 0.71 ± 0.04 h,[107] which is only about 58% of the value found in

the present study. It is interesting that the low pH did not improve the stability of the anthocyanins, especially compared to a study of single strength elderberry juice cooked at 95 °C at pH 3.5, in which cyn 3-sam had a half-life of 1.87 ± 0.07 h while cyn 3-glu had a half-life of 1.96 ± 0.07 h, values that are more similar to our results.[144] In another study on elderberry anthocyanin stability in a commercial concentrate cooked at 95 °C for 6 h, the overall anthocyanin half-life value was 1.96 ± 0.06 h, which is higher than the values in the current study.[145] In another study on elderberry products undergoing thermal processing, the half-life in hours of anthocyanins in pasteurized elderberry juice that contained additives such as sucrose, xanthan gum, potassium sorbate, and ascorbic acid was 26.25 ± 1.49 h at 70 °C, 8.03 ± 0.78 h at 80 °C, and 1.60 ± 0.11 h at 90 °C.[146] While the half-life at 70 °C is significantly higher than the values found in the present study at 72 °C, the half-life at 90 °C is similar to what we observed at 95 °C.

Table 2. Kinetic parameters for anthocyanins in blue elderberry juice

	rate constant (k)		half-life (h)		R^2	
temperature (°C)	72	95	72	95	72	95
cyn 3-sam	0.0017	0.0094	6.80	1.23	0.979	0.997
cyn 3-glu	0.0022	0.0087	5.25	1.33	0.953	0.995

Because elderberry has predominantly cyanidin-based anthocyanins, protocatechuic acid is typically found as the main degradation product, though phloroglucinaldehyde can also be formed.[145] However, neither protocatechuic acid nor phloroglucinaldehyde were observed in any of the cooked juice samples. Protocatechuic acid dihexoside, which was tentatively identified in an earlier study of blue elderberry[39] did not increase over the cooking period. Caffeic acid, a hydroxycinnamic acid (λ_{max} at about 330 nm) increased up to 108.1% of its initial concentration after 2 hours of cooking at 72 °C, and up to 147.1% after 2 hours of cooking at 95 °C. The levels

of caffeic acid were highly variable, with larger standard deviations that the other phenolic compounds. This is a known metabolite of cyanidin-based anthocyanins,[147] and further work investigating the breakdown of anthocyanins in blue elderberry juice into this phenolic acid can elucidate the pathway to this compound.

The main flavonols in blue elderberry, rutin and isorhamnetin glucoside, were stable during the thermal processing, retaining 100.5% and 99.3%, respectively, of their original concentration even at 95 °C (Figure 6). The high retention rates of rutin and isorhamnetin glucoside match literature reports for the thermal stability of these compounds, which show that rutin has a strong thermal stability at acidic pH. More than 80% of the starting concentration was retained after five hours of cooking at 100 °C at pH 5.[148] Our results do not agree with another study in which rutin had an activation energy 107.3 kJ/mol, and the half-life values at 70 and 90 °C were 19.25 and 1.99 h, respectively; however, the rutin was in an aqueous solution at pH 6.6.[149] Other compounds present in blue elderberry juice, in addition to a lower pH, could cause synergistic effects to improve stability of rutin in the present study. Limited information on the thermal stability of isorhamnetin glucoside was found, though a study of black currant juice stability found that during long-term storage at room temperature and at 4 °C, isorhamnetin glucoside concentrations did not change significantly during the 12-month period. In the same study, rutin did not change significantly during storage.[150]

The main phenolic acid in blue elderberry juice, chlorogenic acid, was also thermally stable. This result was unexpected, as another study on the thermal stability of chlorogenic acid in a complex with amylose showed a significant decrease in content after 10-15 minutes, depending on the temperature.[151] Their results also showed that a 10 °C increase in temperature results in a 2.5-fold increase in the rate of degradation of chlorogenic acid. It can be beneficial to maintain

levels of chlorogenic acid in anthocyanin-rich matrices, as shown in black carrot extract where chlorogenic acid increased absorbance of cyanidin-based anthocyanins at pH 3.6 and 4.6 due to intermolecular co-pigmentation.[152]

Overall, our results show that blue elderberry juice behaves similarly to anthocyanin-rich matrices, in that longer processing at higher temperatures degrades anthocyanins. The two main anthocyanins in blue elderberry, cyn 3-sam and cyn 3-glu, behaves similarly during processing, degrading at about the same rate at 72 °C and 95 °C. Furthermore, the other major phenolic compounds like rutin, isorhamnetin, and chlorogenic acid, were highly stable and can withstand the thermal processing.

Our study into the effects of thermal processing on the phenolic composition and cyanogenic glycoside content in blue elderberry juice showed that the main anthocyanins present degrade faster at higher temperatures but other important phenolic compounds like rutin and isorhamnetin 3-glucoside are more thermally stable, retaining over 90% of their original concentrations even after two hours at 95 °C. Furthermore, neoamygdalin and sambunigrin were measured in the blue elderberry juice, which were in lower concentrations compared to European and American elderberry.

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Conflicts of interest

The authors declare no competing financial interest.

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Author contributions

K.R.U.: methodology, conducting analyses, writing the first manuscript draft, and editing.

G.H.C.: conducting analyses and writing.

A.E.M.: project conceptualization, funding, administration, experimental design, supervision, and editing and reviewing the manuscript.

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