International Journal of Fruit Science

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/wsfr20

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To cite this article: Zhuoyu Wang, Andrej Svyantek, Zachariah Miller & Aude A. Watrelot (2024) Influences of Processing Methods on Elderberry (*Sambucus nigra* L.) Wine Quality, International Journal of Fruit Science, 24:1, 389-403, DOI: 10.1080/15538362.2024.2442566

To link to this article: https://doi.org/10.1080/15538362.2024.2442566

Published online: 15 Dec 2024.

INTERNATIONAL JOURNAL OF FRUIT SCIENCE
2024, VOL. 24, NO. 1, 389–403
https://doi.org/10.1080/15538362.2024.2442566

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Influences of Processing Methods on Elderberry (*Sambucus nigra* L.) Wine Quality

Zhuoyu Wang[a], Andrej Svyantek[a], Zachariah Miller[a], and Aude A. Watrelot[b]

[a]Western Agricultural Research Center (WARC), Montana State University, Corvallis, Montana, USA; [b]Department of Food Science and Human Nutrition, Iowa State University, Ames, Iowa, USA

ABSTRACT

Elderberry fruit is available in the market and is becoming more popular. However, winemaking techniques are under-studied with this fruit. This study aimed to determine the impact of three processing methods on elderberry wine quality. The methods were: No extended maceration followed by hot water treatment (EC0); 2 days of cold soak maceration followed by hot water (EC2) and boiled elderberry (EB) fruits before alcoholic fermentation. The results indicated that although the treatments did not cause significant differences in ethanol, glycerol, total acidity, volatile acidity and tannins, wine pHs were influenced by the treatments. Besides influencing the pH, EB treatment produced darker elderberry wine, whereas, EC2 wine had the lightest color. Treatments also influenced the final wine monophenol profiles and antioxidant capabilities. EB wine had the highest total anthocyanin content and antioxidant activities. EC2 wine had a significantly higher amount of total hydroxycinnamates compared to EC0 but was not different from EB-treated wine. The wine antioxidant capacities were significantly lower than in musts but were not different between treatments. Cold maceration in EC2 did not help the extraction of antioxidants in elderberry wine. The results indicated that heating through boiling might help disrupt fruit cells and assist in the extraction of anthocyanins. Cold soak enhanced certain compounds in elderberry wines. This research provided information for elderberry fruit wine fermentation with general processing methods.

KEYWORDS

Elderberry; fruit wine; maceration; cold soak; boiling

Introduction

Elderberry (*Sambucus nigra*) is an anthocyanin-rich fruit that achieved public notoriety during the COVID-19 pandemic due to its potential antiviral properties (Asgary and Pouramini, 2022). *Sambucus spp*. is grown in Australia, Asia, North America, and Africa. There are a diverse number of elderberries, including the North American species *S. nigra* var. *canadensis*, and the European species *S. nigra* var. *nigra* mainly. Other American species (*S. cearulea*) and European species (*S. ebulus*) also exist but are less widely cultivated. The elderberry has been used in folk medicine to treat many diseases and it is well-known for its effects on certain respiratory diseases (Sidor and Gramza-Michałowska, 2015).

Elderberry fruit contains roughly 68.53 to 104.16 g/kg FW sugars and fructose and glucose are the most abundant sugars (Młynarczyk et al., 2018). Elderberry fruit also contains an abundant array of organic acids; mainly citric acid, malic acid, shikimic acid, and fumaric acid (Veberic et al., 2009). Beyond the primary metabolites in elderberry fruit, the berries are particularly rich in flavonoid compounds, including anthocyanins, proanthocyanidins, as well

CONTACT Zhuoyu Wang zhuoyu.wang@montana.edu Western Agricultural Research Center (WARC), Montana State University, 580 Quast Ln, Corvallis, MT 59828, USA

as flavonols. Anthocyanins are the most important polyphenols driving the dark color of elderberry fruit (Cejpek et al., 2009). Elderberry fruit contains many polyphenolic compounds with antioxidant capacities such as flavonols and anthocyanins (Liu et al., 2022). The fruit antioxidant capacity ranks higher than some well-known fruits, such as blueberry and cranberry (Cejpek et al., 2009; Prior et al., 2002). Elderberry flowers and fruits also contain valuable micronutrients and minerals (Młynarczyk et al., 2020). "Samyl" elderberry is a cultivar that belongs to *S. nigra* and it is among the most popular elderberry cultivars; its flowers and fruits have substantial amounts of minerals, bioactive compounds, and high antioxidant activity (Csorba et al., 2020).

Elderberry can be processed into many food products with pleasant aromas, tastes, and complex compositions (Auzanneau et al., 2018). Elderberry wine as a value-added product is reported to have high biological and industrial potential (Terzić et al., 2022). Despite this potential, there is limited research available for elderberry wine fermentation. Elderberry wine from European species has been fermented, revealing a high correlation between anthocyanin content and elderberry wine color (Schmitzer et al., 2010). Different temperature treatments' effects on elderberry fermentation were studied in Serbia with results indicating that different temperature treatments had different pharmacological content extraction effects; the highest temperature (70°C) extraction wines had the highest antioxidant activity while the 60°C treated wines had the best inhibitory effect against β- glucosidase (Terzić et al., 2022). Elderberry fruit wine production has been studied based on different combinations of sugar and water content in Croatia; wines with high sugar quantities and low acidity were preferred by consumers (Garofulić et al., 2012). Elderberry wine has been compared with blackberry, cranberry, bilberry, raspberry, and strawberry wines; the results indicate that among these fruit wines, elderberry wine is the richest source of polyphenols and contains the greatest concentration of hydroxycinnamic acids, anthocyanins, and flavonols (Czyżowska et al., 2020). Similarly, elderberries produced the highest values of antioxidant compounds compared with apple, strawberry fruit wines, and some grape wines (Cehula et al., 2020). Elderberry extract and various fruit juices have been examined to optimize the overall wine aroma and flavor (Cao et al., 2023). Elderberry wine with its unique characteristics, such as high pH (3.9–4.17) and low acidity, requires continued research to optimize fermentation, compound extraction, and chemistries. As in grape wine fermentation, many factors affect wine aroma and taste during fruit wine fermentation (He et al., 2023). The main factors impacting wine include fruit growing environment, input materials, and fermentation process (He et al., 2023; Yang et al., 2021). Elderberry variety selections also play an important role in elderberry wines' physical characteristics and volatile compounds during fermentation (Serviss et al., 2024).

Due to the potential toxicity of elderberry (fruit release cyanide compounds), fermentation techniques shall also consider this aspect. It has been reported that human consumption of cyanogenic plants, such as elderberries, can cause sub-acute cyanide poisoning with symptoms of anxiety, headache, vomiting, nausea, diarrhea, dizziness, mental confusion, and even death (Geller et al., 2006). Food processing techniques, such as drying, grinding, and heating, can be used to reduce the potential toxicity of plants containing cyanogenic glycosides (Gleadow et al., 2012; Senica et al., 2016). Elderberry fruit is often mixed with hot boiling water and cooled down before yeast inoculation for wine fermentation (Schmitzer et al., 2010). Along with sugar and water additions, research on elderberry wine has assessed the addition of other fruit juice and inoculation via different yeast strains (Cao et al., 2023; Garofulić et al., 2012; Tokar et al., 2021). Beyond this, there is limited information available on elderberry wine fermentation strategies in research. Therefore, in this research, we compared boiling with different times of cold soaking combined with hot water mixed as pretreatments in elderberry wine fermentation.

Maceration is a technique of soaking fruit skins with fruit juice in combination, with or without agitation, for some period of time with the goal of extracting more color and aroma compounds (Irfan et al., 2022). Pre-fermentative cold maceration, known as cold soaking, can enhance polyphenol contents, color intensity, and sensory attributes in wines (Bestulić et al., 2022; Lasanta et al., 2023).

This potentially beneficial technique has not been examined as a part of elderberry fermentation. This study is the first research to compare cold soak and hot maceration for their impacts on elderberry fermentation. Meanwhile, cooked elderberries were compared with the maceration techniques in elderberry wine fermentation.

Materials and methods

Plant materials and fermentation procedures

Elderberry fruit from "Samyl" was grown at the Western Agricultural Research Center of Montana State University, Corvallis, MT. Fruits were hand-harvested in September 2022 and frozen in a walk-in freezer at −20°C. Two days before fermentation, approximately 10 kilograms of frozen elderberries were transferred into a walk-in cooler with temperatures of 0°C to 4°C and humidity of 90% to 95%. On the day of fruit processing, elderberries were crushed in a basket with a potato masher. Elderberries were then separated for each treatment as 1 kg crushed elderberries with 3 L of drinking water; treatments were conducted in triplicate for three replicate fermentations. Three treatments were carried out as (1) boiling (EB): elderberries were added to three liters of water and boiled for five minutes; (2) hot water (EC0): crushed elderberries were added to three liters of hot water (100°C); (3) two days of cold soaking followed by hot water treatment (EC2): One liter of drinking water (at room temperature) and 1 kg elderberries were combined in each replicate and stored in a walk-in cooler for two days. For the EC2 treatment, Gaïa^{TM} (IOC, France) was added into the macerated must to reduce bacteria growth. After two days, two liters of hot water (100°C) was added to the must (Figure 1). All the must in each treatment was cooled to room temperature (25°C) before pressing. Elderberry must

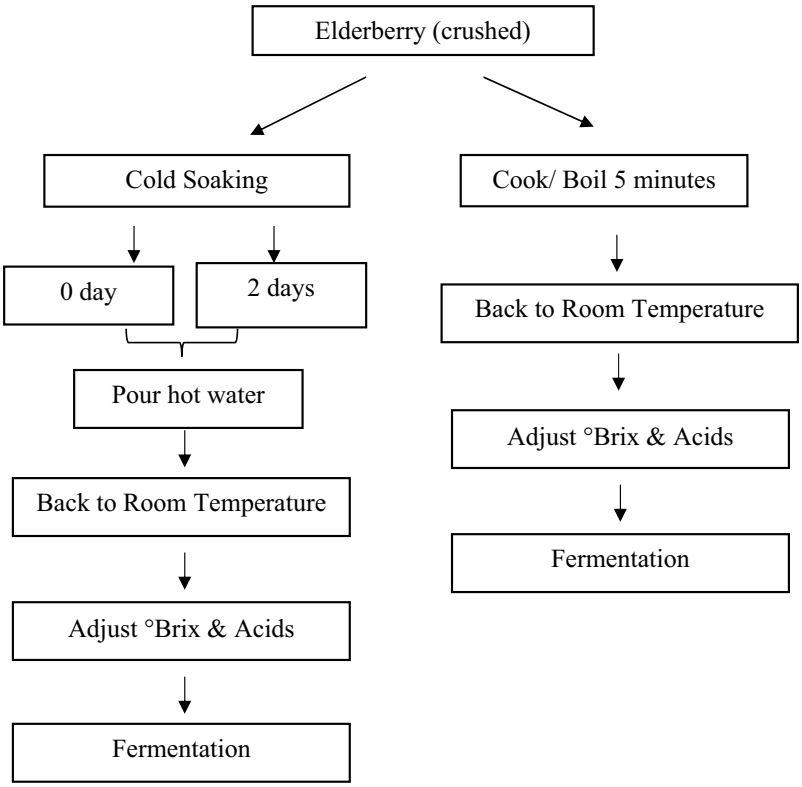

Figure 1. Elderberry fermentation flow chart. Three treatments are (1) 5 minutes of boiling (EB); (2) No cold soaking (0 day) followed by hot water (EC0); (3) two days of cold soaking followed by hot water (EC2).

was obtained using a 6 L hydraulic fruit press (SQUEEZE master, USA) with a pressure of 2 Bar. For each replicate, 3.5 L of must was poured into an individual fermenter (Little Big Mouth Bubblers; Northern Brewer, Roseville, MN, USA). A mixture of 25 mg/L potassium metabisulfite (Northern Brewer, US), 0.5 g/L Lysozyme (Scott Labs, Santa Rosa, CA, USA), 0.5 g/L Bactiless (Scott Labs, Santa Rosa, CA, USA), and 350 mg/L of Scottzyme HC (Scott Labs, Santa Rosa, CA, USA) was added into each fermenter and homogenized thoroughly via mixing. The next day, soluble solid content (SSC, ° Brix) was tested through an Anton Paar DMA 35 V4 digital density meter (Anton Paar, Torrance, CA, USA). Corn sugar (Brewmaster, Pittsburg, CA, USA) was added into each fermenter to adjust °Brix to the target value of 17. Meanwhile, the pH of each must was tested through an ATAGO PAL-pH meter (ATAGO, Tokyo, Japan). Must pH was then adjusted to approximately 3.75 via the addition of malic acid (Cellar Science, Pittsburg, CA, USA). Online software, FermCalc (https://fermcalc.com/FermCalcJS.html), was used to assist with the sugar and malic acid measurement for adjusting °Brix and pH. For primary fermentation, EC-1118 (Lalvin, Canada) yeast strain was added to musts at an inoculation rate of 0.264 g/L after rehydration in Go-Ferm Protect Evolution™ (Scott Labs, Santa Rosa, CA, USA) according to the manufacturer's manuals. On the third day of each fermentation, 0.5 g/L Fermaid® O yeast nutrient (Northern Brewer, USA) was added to each fermentation vessel. The total primary fermentation lasted approximately 10 days. After completion of alcoholic fermentation, wines were manually racked off their lees into sanitized, 1.89 L clear glass fermenters (ULINE, Pleasant Prairie, WI, USA) fitted with airlocks. Wines were stored in these containers for approximately two months before botting. At bottling, wines were manually transferred via gentle pouring into 187 mL clear glass bottles with the addition of sulfur at a rate of 40 mg/L potassium metabisulfite before capping with oxygen-scavenging crown caps.

Sample collection

Throughout the first 10 days of primary fermentation, samples were collected daily from each fermenter using individual, sterile, serological pipets. The samples were transferred into 15 mL centrifuge tubes and stored immediately in a −20°C freezer until analysis. Final wine samples were collected five months after bottling and transferred into argon-flushed, 29.57 mL Amber Boston Round Glass bottles (ULINE, Pleasant Prairie, WI, USA).

Sample analysis

Pre-fermentation must samples were analyzed for total Yeast Assimilable Nitrogen (YAN_{Total}) nutrients through Primary Amino Nitrogen (PAN) analysis with a K-PANOPA kit (Neogen, US) and L-Arginine/Urea/Ammonia YAN_{Aug} analysis (K-LARGE, Neogen, US). The equation to calculate total YAN was as follows: YAN_{Total}= YAN_{Aug}+PAN. Soluble solid content (°Brix) was monitored using density meter DMA35 (Anto Paar, US) through the fermentation processes. pH was assessed using an Atago portable pH meter (PAL-pH; Atago Co., Japan). The tannin content of the final wine was tested using a Tannin microplate assay kit (MyBioSource, San Diego, CA, USA).

During fermentation, color parameters were monitored across the visible spectrum, recording from 380 nm to 700 nm with 1 nm interval) via a SPECTROstar^{nano} microplate reader (BMG Labtech, US). The color data was converted by the software ColorBySpectra (Farr and Monica Giusti, 2018). Then the absorbance data was translated into CIEL*a*b color space with the standard of Illuminant D65. The color data was reported with parameters of L* (lightness), a* (green-red), and b* (blue-yellow). L*, a*, and b* dynamic changes during fermentation were plotted by R 4.2.1. Core Team (2023) with the ggplot2 package. Color differences between must and wine were calculated with the formula $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ (Yuan et al., 2007).

The wine ethanol, glycerol, and volatile acidity concentrations were analyzed in final elderberry wines by an FTIR wine analyzer (Lyza5000 wine, Anton Paar, US). The titratable acidity of the final wine was detected according to AOAC methods (Horwitz and Latimer, 2005; Lee et al., 2005). The

results were calculated as a percent (grams of malic acid equivalent/100 g). The equation was $TitratableAcidity(\%) = \frac{0.1NNaOH \times 0.0067}{mlof\ wineused} \times 100$.

Analysis of monomeric phenolic concentrations by HPLC-DAD

Monomeric phenolic compounds of elderberry wines were analyzed using a 1260 Infinity II HPLC (Agilent Technologies, Santa Clara, CA, USA) with a reverse-phase column (LiChrospher 100–5 RP18 250 mm × 4.0 mm, 5 µm, Agilent Technologies), DAD (Agilent 1260 Infinity II DAD WR) (Agilent 1260 Infinity II FLD Spectra) as the methods in publications (Gómez-Alonso et al., 2007; Ritchey and Waterhouse, 1999). The mobile phases were 50 mm ammonium dihydrogen phosphate pH 2.6 (mobile phase A), 20% (v/v) mobile phase A in acetonitrile (mobile phase B), 0.2 M orthophosphoric acid in the water, pH 1.5 (mobile phase C). The detailed gradient followed the previous publication (Watrelot et al., 2018). The column temperature was maintained at 40°C with a flow rate of 0.5 mL/min. An amount of 20 µL of sample supernatant was injected. The monomeric phenolics were identified and quantified at different wavelengths: 280 nm for flavanols, 316 nm for hydroxycinnamic acids, 360 nm for flavonols, and 520 nm for anthocyanins. Flavan-3-ols were quantified using (-)-epicatechin as the reference standard. Hydroxycinnamic acids were quantified using caffeic acid as the reference standard. Flavanols were quantified using quercetin-3-O-glucoside as standard. Anthocyanins were quantified using cyanidin-3-O-glucoside as the standard.

Elderberry must and wine antioxidant activities assays

The antioxidant activity of elderberry must and wine samples was analyzed through the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the Ferric Reducing Antioxidant Power (FRAP) assay. Following the manual of the DPPH antioxidant assay kit (ab289847, Abcam, Cambridge, UK), the results of DPPH activities were represented as Trolox Equivalent Antioxidant Capacity (TEAC eq. µM/µL). The FRAP assay was tested via a colorimetric FRAP assay kit (ab234626, Abcam, UK) following the manufacturer's instructions and the results were expressed as mM Ferrous (Fe^{2+}) equivalents.

Statistical analysis

All experimental data were analyzed with R 4.2.1 (R Core Team, 2023) and expressed as mean ± standard deviation (SD). One-way ANOVA was performed for each treatment for the final wines, assessing treatment as the main effect and replicate as a random effect. The lme4 1.1–31 package was used for fitting and analyzing models, emmeans 1.8.5 package was used to separate the least-square mean values (Lenth, 2016; Searle et al., 1980).

Table 1. Total soluble solids content and pH of musts immediately before and immediately after sugar and acid adjustments for elderberry musts based on three pre-fermentative must treatments.

	Immediately before must adjustment		Immediately after must adjustment	
Treatment	SSC (°Brix)	pH	SSC (°Brix)	pH
EC0	2.4 ± 0.1[a]	4.33 ± 0.02[a]	16.6 ± 0.1[a]	3.66 ± 0.03[a]
EC2	2.4 ± 0.1[a]	4.30 ± 0.02[a]	16.7 ± 0.2[a]	3.85 ± 0.04[b]
EB	2.5 ± 0.1[a]	4.33 ± 0.02[a]	16.9 ± 0.2[a]	3.63 ± 0.04[a]

Elderberry fermentation followed three must pre-treatments: hot water treatment (EC0); cold soak with *M. fructicola* inoculum followed by hot water treatment (EC2); and boiling of fruit (EB). Each treatment contains three replicates. Each value is listed as the mean ± standard error of replicates. Treatments with the same letter within columns are not significantly different according to Tukey HSD test at α = 0.05.

Results and discussions

Elderberry must composition

Elderberry musts had very low initial SSC (2.4–2.5 °Brix) and high pH (3.63–3.85) (Table 1). From the original elderberry must sugar content, with an SSC of only 2.4–2.5, it was evident that elderberry must mixed with water alone lacked the necessary levels of fermentable sugars for standard wine fermentation. The elderberry fruit and water ratio prior to pressing was approximately 1:3. Therefore, elderberry juice sugar content from the berries alone would be approximately quadruple the °Brix in our fermentation must, approximately 10 °Brix. The SSC is close to the elderberry fruit composition observed in prior work, indicating approximately 10.5 °Brix for elderberry fruits (Perkins-Veazie et al., 2015).

Beyond fermentable sugars, pH is considered a critical factor in regulating wine fermentation, influencing yeast and enzyme activities, and altering a spectrum of other production considerations (Gambuti et al., 2022; Vicente et al., 2022). The initial high pH (~4.3) was not optimum for fermentation stability and yeast fermentation efficiency (Czibulya et al., 2015; Sims and Morris, 1985). This research used malic acid to decrease the pH below 4 to improve the must stability. Although the same amount of sugar and acids were added due to the initial SSC and pH values, the final pH following adjustment had only minor variation. The pH in EC2 (approximately 3.85) was higher than the other two treatments, which were approximately 3.60. The SSC did not have significant differences ($p < .05$) after sugar adjustment, ranging from 16.6 to 17.0.

Nitrogen is essential for yeast fermentation, and the nitrogen sources can influence the fermentation process (Gobert et al., 2019). In this study, nitrogen sources were dominated by primary amino nitrogen (PAN) content as the largest component in YAN_{Total} for each treatment (Table 2) before yeast inoculation. Values ranged from about 83% (25.73/31.81 mg of N/L) in EC2 to 95% (45.45/47.63 mg of N/L). There were no significant differences in PAN, YAN_{Aug} and YAN_{Total} content between EB and EC0 before yeast inoculation, but both had significant ($p < .05$) higher amounts of these nitrogen sources compared to EC2 before yeast inoculation. EC2 had only about half the amounts of PAN content (about 25.73 mg of N/L), but higher amounts of YAN_{Aug} (6.51 mg of N/L) compared to EC0 (3.17 mg of N/L) and EB (2.17 mg of N/L).

$YAN_{Aug,}$ nitrogen derived from L-arginine, urea and ammonia was detected in greater quantities in EC2 treatments during maceration, from 6.51 to 9.50 mg of N/L with significant differences ($p < .05$). It may stem from enhancement of YAN_{Aug} extraction during *Gaïa* maceration or enhancement of YAN_{Aug} via the addition of *Gaïa* inoculum in comparison to EB and EC0 treatments.

Based on the elderberry must nitrogen content, musts without maceration (EC0) had similar quantities of yeast assimilable nitrogen (YAN) amount as the boiled treatment; the YAN ranged from about 47 to 53 mg N/L (Table 2). With maceration, EC2 treatment showed only half of the nitrogen (about 31.81 mg of N/L) at the beginning. After two days of maceration, the level of total

Table 2. Primary amino nitrogen (PAN) and yeast available nitrogen (YAN; composed of arginine, urea, and ammonia) content for three elderberry musts with three pre-fermentative must treatments at multiple time points before *Saccharomyces* yeast inoculation.

Must time point	Treatment	PAN (mg of N/L)	YAN_{Aug} (mg of N/L)	YAN_{Total} (mg of N/L)
Before *Saccharomyces* yeast inoculation	EB	45.45 ± 1.64^{c}	2.17 ± 0.10^{a}	47.63 ± 1.56^{c}
Before *Saccharomyces* yeast inoculation	EC0	50.60 ± 1.49^{c}	3.17 ± 0.69^{a}	53.87 ± 2.18^{c}
Before *M. fructicola* inoculation	EC2	25.73 ± 4.49^{b}	6.51 ± 0.36^{b}	31.81 ± 4.83^{b}
1 day after *M. fructicola* inoculation	EC2	25.08 ± 3.49^{b}	7.19 ± 0.78^{bc}	32.27 ± 4.27^{b}
2 days after *M. fructicola* inoculation, immediately before *Saccharomyces* yeast inoculation	EC2	16.49 ± 1.51^{a}	9.50 ± 0.96^{c}	25.99 ± 2.43^{a}

Elderberry fermentation followed three must pre-treatments: hot water treatment (EC0); cold soak with *M. fructicola* inoculum followed by hot water treatment (EC2); and boiling of fruit (EB). Each treatment contains three replicates. The EC2 must was monitored at three time points due to on-going, cold soak maceration in the presence of *M. fructicola*. Each value is listed as the mean ± standard error of replicates. Treatments with the same letter within columns are not significantly different according to Tukey HSD test at $\alpha = 0.05$.

nitrogen content was close to the initial YAN_{Total} without significant differences ($p < .05$), only YAN_{Aug} was enhanced with 9.50 mg of N/L with maceration. This may indicate that *Gaïa* consumed some nitrogen during the maceration stages. *Gaïa*, a *Metschnikowia fructicola* yeast strain has not been evaluated for YAN requirements. In this research, compared to the other two treatments, *Gaïa* consumed more than half of the elderberry must's native YAN before fermentation, leaving only about 25.99 mg N/L nitrogen for potential fermentation usage.

Total soluble solids content changes during alcoholic fermentation

Elderberry fermentation dynamic changes (Figure 2) were similar among the three treatments. Without maceration, EC0 and EB had a similar SSC consumption rate, with °Brix decreasing at a steady pace. The EC0 had a slightly faster °Brix decrease compared to the EB treatment. EC2 had slightly increased °Brix (about 2.5) between day 0 to day 1 during the maceration stages, indicating more sugar was dissolved into musts. On day 2, with more corn sugar added, SSC (approximately 17 °Brix) was the same as the other two treatments after the sugar adjustment. Similarly, the sugar dynamic changes were roughly the same as the other two treatments with the similar slopes (−2.05 to −2.2), which indicated that the dynamic changes were mainly related to yeast additions. The cold soaking and temperature-aided extraction employed in this study did not alter the SSC consumption fermentation dynamic changes. From other studies, it indicated that cold soaking with simultaneous yeast inoculation could modify the sugar consumption rate by combining low temperature and lower oxygen dissolved in the must (Casassa and Sari, 2015). In this study, since elderberry juice was pressed and sugar was adjusted after soaking, there was no indication of a dramatically modified sugar consumption rate and sugar reduction rate from the highest (~17 °Brix) to the lowest SSC (≤0 °Brix).

Elderberry fermentation color dynamic parameters

Elderberry must treatments were important in differential color dynamics (Figure 3). Mainly, fermentation via different treatments was altered in lightness across fermentation time. EC0 had

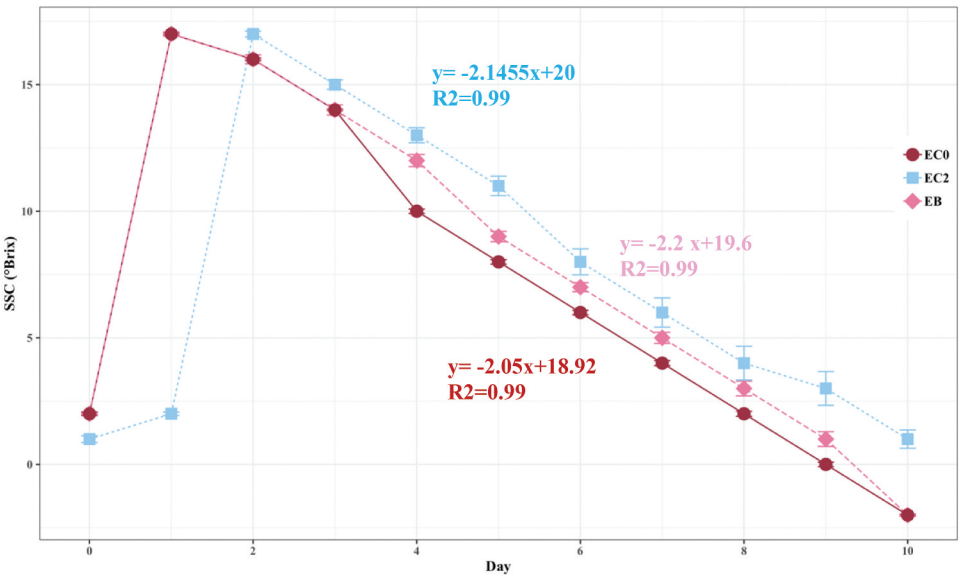

Figure 2. Soluble solid content (SSC) dynamic changes during fermentation of elderberry wines produced following three pre-fermentative must treatments: hot water treatment (EC0); cold soak with *M. fructicola* inoculum followed by hot water treatment (EC2); and boiling of fruit (EB). The standard deviations of sample means within each sampling day are depicted as standard error bars. Equations on the chart indicated the SSC reduction trendline from the highest °brix (17) to the lowest °brix (≤0). The equation colors are corresponded to the treatment legend colors.

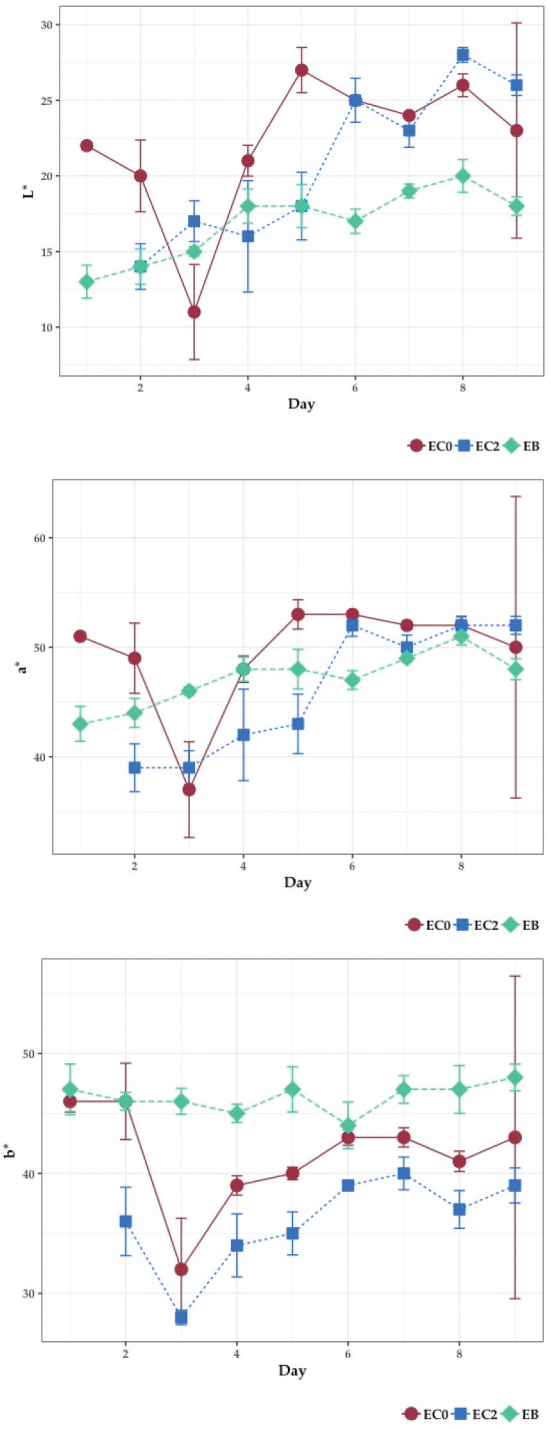

Figure 3. Elderberry must color dynamic changes during fermentation of elderberry wines produced following three pre-fermentative must treatments: hot water treatment (EC0); cold soak with *M. fructicola* inoculum followed by hot water treatment (EC2); and boiling of fruit (EB). CIELAB color space parameters depicted are L* (lightness); a* (red/green color parameter values); b* (blue/yellow color parameter values). The standard deviations of sample means within each sampling day are depicted as standard error bars.

Table 3. Elderberry fermentation start must and final wine color.

	Start Must				Final Wine				Differences
Treatment	L*	a*	b*	Color	L*	a*	b*	Color	ΔE
EC0	21.75	50.99	46.22		16.00	36.84	29.80		251.42[c]
EC2	14.38	40.56	36.57		25.90	51.06	38.57		123.42[b]
EB	12.92	42.68	45.74		17.97	47.89	47.48		27.83[a]

Average LAB indexes (L*, a*, b*) are listed. The representative must and wine color are filled within the Color column. Color input follows C 2° illumination and reference angle. The total color differences of each treatment were calculated, and the results were listed as ΔE. Treatments with the same letter within columns are not significantly different according to Tukey HSD test at α = 0.05.

dramatically decreased lightness on day 3 during fermentation, which might indicate the maximization of color compounds in the must solution on day 3. The final sampled elderberry wine lightness showed that boiled elderberry extraction (EB) was the darkest based on L* values; however, at the completion of fermentation, large, overlapping, variations within samples of EC0 remained.

Initially, musts were dramatically different based on a* values, which shifted across fermentation dates. After finishing primary fermentation, all the treatments had similar a* values (around 50). This indicated that variation was reduced among treatments within the red/green color parameters.

EC2 had the lowest b* values during fermentation. EC0 had a similar b* index changing patterns as b* in EC2 treatments, although its b* values were higher than EC2 on all sampling dates. The b* values varied the least in EB treatment during fermentation, ranging from 45 to 50.

Overall, based on LAB color changes (Table 3), must changes showed the least dramatic changes in EB treatment during fermentation with ΔE about 27.83, followed by EC2 with ΔE value of 123.42. EC0-treated must had the largest changes with ΔE of 251.42 during fermentation. This may be due to the concentration of pigment compounds in EB must or potential homogenization of musts, both stemming from added breakdown of fruit tissue which may have occurred during boiling of fruit.

Final wine characteristics

From the final wine characteristics (Table 4), EC0 and EC2 had similar pH values (around 3.50). EB treatment had a lower pH value (3.48), although it was not significantly different from EC0. Ethanol content did not differ among these three treatments; all treatments were just below 10% ethanol in the final wines. Glycerol content was also similar for the three treatments (above 5 g/L glycerol in the final wines). These characteristics indicate that the pretreatment strategies did not alter the main characteristics of the final wines. In other words, the elderberry viscosity was not affected by the treatments, since the ethanol and glycerol contents were not significantly modified by the treatments (Yanniotis et al., 2007).

The titratable acidity was approximately 4.9–5.0 g/L and the tannin was approximately 0.4–0.46 g/L in the final wines (Table 5). There were no significant differences detected among the treatments. For grape wine sensory perception, titratable acidity offers the tartness. In this study, the titratable acidity levels were consistent with typical ranges of grape wine titratable acidity (4-8 g/L). However, little

Table 4. Final wine pH, ethanol, and glycerol for three elderberry wines produced following three pre-fermentative must treatments.

Treatment	pH	Ethanol (%vol)	Glycerol (g/L)
EC0	3.51 ± 0.01[ab]	9.77 ± 0.04[a]	5.47 ± 0.15[a]
EC2	3.52 ± 0.02[b]	9.78 ± 0.17[a]	5.87 ± 0.25[a]
EB	3.48 ± 0.01[a]	9.98 ± 0.13[a]	5.53 ± 0.25[a]

Elderberry fermentation followed three must pre-treatments: hot water treatment (EC0); cold soak with *M. fructicola* inoculum followed by hot water treatment (EC2); and boiling of fruit (EB). Each treatment contains three replicates. Each value is listed as the mean ± standard error of replicates. Treatments with the same letter within columns are not significantly different according to Tukey HSD test at α = 0.05.

Table 5. Final wine titratable acidity, volatile acidity, and tannin content for three elderberry wines produced following three pre-fermentative must treatments.

Treatment	Titratable acidity (g/L)	Volatile acidity (g/L)	Tannin content (g/L)
EC0	4.91 ± 0.07[a]	0.55 ± 0.02[a]	0.45 ± 0.02[a]
EC2	4.90 ± 0.37[a]	0.59 ± 0.03[a]	0.46 ± 0.10[a]
EB	5.03 ± 0.15[a]	0.62 ± 0.05[a]	0.40 ± 0.02[a]

Elderberry fermentation followed three must pre-treatments: hot water treatment (EC0); cold soak with *M. fructicola* inoculum followed by hot water treatment (EC2); and boiling of fruit (EB). Each treatment has three replicates. Each value is listed as the mean ± standard error of replicates. Treatments with the same letter within columns are not significantly different according to Tukey HSD test at α = 0.05.

research has addressed the importance of titratable acidity to the sensory evaluation for elderberry wines (Warmund et al., 2016). Tannin is mainly found in the skin, stems, and seeds of fruits and the content in elderberry fruit can exceed 1 g/L (Schmitzer et al., 2010). Considering the fruit ratios (1 kg in 3 L of water) used in this study, the tannin extraction was effective, and its content will provide astringency in the final wines.

A low level of volatile acidity (0.55 to 0.62 g/L) was detected in the final wine. Volatile acidity is not favorable for wine consumers and the control of it is a major issue for wine quality (Z. Luo et al., 2013). In the elderberry wines, volatile acids were present in small amounts, far less than the limits for red and white wines, which are 1.2 to 1.4 g/L in the U.S.A (Zoecklein et al., 1995). Therefore, it will not be problematic in the elderberry wines produced in this study.

Elderberry wine monomeric phenolic concentrations

Across the four major groups of phenolic compounds evaluated, anthocyanins were the most abundant, with more than 138 mg/L in each wine (Table 6). Two days of cold soaking did not enhance the anthocyanin extraction. Surprisingly, the total anthocyanin quantity in EC2 wines (138.25 mg/L) was lower than the other two treatments (>170 mg/L); significant differences were detected between EC2 and EB. EB treated wine contained the highest anthocyanin content (211.68 mg/L). These results aligned with the relatively higher L* and lower b* values compared to the other treatments after primary fermentation (Figure 3). In contrast, EB wines showed the highest concentration of anthocyanins and had, correspondingly, the lowest L* values. Anthocyanins, a group of natural pigments, are responsible for the red-blue color in fruits (de Pascual-Teresa and Teresa Sanchez-Ballesta, 2008). The anthocyanins contents also explained the corresponding wine colors (Table 3). EC2 and EB with high levels of anthocyanins shower darker red color, whereas EC2 had brighter red color.

Plant polyphenols as dietary antioxidants are beneficial in human health, and several subgroups, such as anthocyanins and flavonoids, were well-known for their functional benefits (Pandey and Ibrahim Rizvi, 2009). Hydroxycinnamates, a class of aromatic acids or phenylpropanoids, are the precursors for flavors in the food industry (Kroon and Williamson, 1999). Hydroxycinnamates can provide most of the antioxidant activity observed in many foods, further reducing the disease risk and

Table 6. Final wine total flavanol, hydroxycinnamates, flavonols, and anthocyanin content for three elderberry wines produced following three pre-fermentative must treatments.

Treatment	Total Flavanols (mg/L)	Total Hydroxycinnamates (mg/L)	Total Flavonols (mg/L)	Total Anthocyanins (mg/L)
EC0	125.51 ± 4.80[a]	21.61 ± 1.22[a]	103.42 ± 3.94[a]	178.66 ± 3.15[ab]
EC2	124.46 ± 6.16[a]	25.22 ± 0.25[b]	98.53 ± 24.21[a]	138.25 ± 7.70[a]
EB	145.68 ± 5.20[a]	22.70 ± 0.35[ab]	130.29 ± 2.63[a]	211.68 ± 19.43[b]

Elderberry fermentation followed three must pre-treatments: hot water treatment (EC0); cold soak with *M. fructicola* inoculum followed by hot water treatment (EC2); and boiling of fruit (EB). Each treatment contains three replicates. Total flavanols are expressed as epicatechin equivalent (mg/L); Total hydroxycinnamates are expressed as Caffeic acid equivalent (mg/L); Total flavonols are expressed as Quercetin-3-glucoside equivalent (mg/L); Total anthocyanins are expressed as Cyanidin-3-glucoside equivalent (mg/L). Each value is listed as the mean ± standard error of replicates. Treatments with the same letter within columns are not significantly different according to Tukey HSD test at α = 0.05.

promoting human health (Kroon and Williamson, 1999). The estimated hydroxycinnamates in the fermented elderberry wines ranged from approximately 21 to 25 mg/L (Table 6). The cold soaking maceration conducted for the EC2 treatment resulted in more hydroxycinnamates (25.22 mg/L). Cold soak has been demonstrated to increase the extraction of anthocyanins, tannins, and aroma compounds in grape wines (Pérez-Lamela et al., 2007). Still, mixed results of cold soak efficiency effects have been reported, with studies observing varying results from cold soak on hydroxycinnamates extraction and wine composition (Lerno et al., 2019; Panprivech et al., 2015). Therefore, further research on the impacts of cold soak length and temperature on elderberry wine monophenol extraction may be an interesting future focus for researchers.

The flavonoid subgroups, flavanols and flavonols were detected in the elderberry wines (Table 6), but there were no significant differences among the treatments. The flavanol content was about 124 mg/L to 145 mg/L and the flavonols content was approximately 98 to 130 mg/L in the elderberry wines.

Flavanols have been found in common foods, such as beans, vegetables and fruits. These common secondary plant metabolites exhibit many beneficial properties for human health (Y. Luo et al., 2022). Flavonols, as antioxidants and antiinflammation agents, are also recommended for health maintenance (Wang et al., 2006). It has been reported that food processing techniques, such as heating, can contribute to the degradation of flavonoids (Ioannou et al., 2012). The boiling treatment in EB examined in this study was the treatment that had the most thorough heating of fruits, which might most consistently disrupt the fruit cells of the elderberry. It has also been reported that cooking the musts could induce chemical, physical and functional changes in grape wines, but this effect is related to the length of time boiling (Piva et al., 2008). In this study, there were no significant differences in flavonoids in the elderberry wines, which indicated that five minutes of cooking in EB treatment might not have been sufficient to cause significant degradation of the flavonoid compounds.

Antioxidant activities (DPPH & FRAP assays) in elderberry must and wine

Antioxidant activities of elderberry were reported for its plant tissues, extract, food, supplements, and tea infusions (Sidor and Gramza-Michałowska, 2015). Using the DPPH assay, wine has been previously demonstrated to have more antioxidant activities than must (Schmitzer et al., 2010). In this study, the antioxidant activities (Table 7) in the EB must had the highest antioxidant activities tested by both DPPH and FRAP assays, with more than 1000 TEAC μM/μL and about 289 mm Fe^{2+} /10 μL antioxidant activity. The must from each treatment had more antioxidant capabilities than its corresponding final wine. Following EB treatment, EC0 must had higher antioxidant activities (957.31 TEAC μM/μL by DPPH and 240 mm Fe^{2+} /10 μL by FRAP assays) than EC2 must from day

Table 7. Antioxidant activity of three elderberry musts and wines based on three pre-fermentative must treatments.

Category	Treatment	DPPH (TEAC μM/μL)	FRAP (mM Fe^{2+} /10 μL)
Must	EB	1013.17 ± 6.58^{a}	289.34 ± 6.54^{a}
Must	EC0	957.31 ± 22.29^{a}	240.04 ± 3.01^{b}
Must	EC2_M0	472.52 ± 22.49^{c}	126.89 ± 8.94^{d}
Must	EC2_M1	676.09 ± 25.43^{b}	212.68 ± 12.65^{b}
Must	EC2_M2	648.16 ± 19.68^{b}	209.70 ± 4.42^{b}
Wine	EB	787.82 ± 9.47^{b}	248.69 ± 5.33^{b}
Wine	EC0	765.56 ± 65.66^{b}	174.89 ± 12.96^{c}
Wine	EC2	659.99 ± 10.70^{b}	165.57 ± 9.91^{cd}

Elderberry fermentation followed three must pre-treatments: hot water treatment (EC0); cold soak with *M. fructicola* inoculum followed by hot water treatment (EC2); and boiling of fruit (EB). Each treatment contains three replicates. DPPH assay results are shown as μM/μL Trolox Equivalent Antioxidant Activities. FRAP assay results are shown as mM Ferrous Equivalent (Fe^{2+})/10 μL. EC2_M0 to M2 indicate EC2 must from day 0 to day 2 of cold maceration in the presence of *M. fructicola*. Each value is listed as the mean ± standard error of replicates. Treatments with the same letter within columns are not significantly different according to Tukey HSD test at $\alpha = 0.05$.

0 to day 2 of maceration (about 650 TEAC μM/μL by DPPH and less than 220 mm Fe^{2+} /10 μL by FRAP assays), on average. However, the FRAP assay showed a nonsignificant difference after day 1 of maceration. The principles of DPPH and FRAP assays may cause differential detections of antioxidant activities in must and wine samples. The DPPH assay is based on an antioxidant reaction with free organic radicals. In contrast, the FRAP assay is performed to measure the antioxidant potential through the reduction of Fe^{3+} to Fe^{2+} by antioxidants (Christodoulou et al., 2022).

Within the cold soak maceration process, EC2 must showed the highest antioxidant (676 TEAC μM/μL by DPPH assay and 212.68 mm Fe^{2+} /10 μL by FRAP assay) activities after one day of maceration. Two days of maceration did not improve the must's antioxidant capabilities significantly with two methods.

In elderberry wines, the EB treatment produced the wine with the highest antioxidant capabilities, followed by EC0 and EC2. The DPPH method did not separate the antioxidant activities among elderberry wines, showing wine had a range from approximately 660 to 780 TEAC μM/μL antioxidant activities. The FRAP method indicated significant differences in antioxidant capabilities among the wines from different treatments. The EB treatment wine had nearly 250 mm Ferrous Equivalent/10 μL antioxidant activities; this was 80 mm Ferrous Equivalent/10 μL antioxidant activity greater than the EC2 elderberry wine.

Elderberry wine showed more antioxidant activities in commonly consumed Italian wines than blueberry, blackcurrant, cranberry, plum, apple, peach, and pear wines (Pellegrini et al., 2003). Meanwhile, it has been reported that antioxidant activities were increased in elderberry and other fruit juice blended fermentations (Cao et al., 2023). Within this study, 1 kg fresh weight of elderberry fruit was combined with 3 L of drinking water for each fermenter. The antioxidant activities, in general, indicated that wine decreased antioxidant capabilities compared to the fruit must. Boiling of elderberry showed promising antioxidant and anthocyanin extraction efficiency (Table 6), although boiling might influence the total flavanol and hydroxycinnamate content. Cold maceration across two days did not increase the extraction of antioxidants and reduced the anthocyanin content in the final wine. Hot water treatment, a common elderberry winemaking strategy, resulted in moderate antioxidants and high anthocyanin content compared to the other two methods.

Boiling or blanching is frequently used for tropical and subtropical fruits before wine fermentation (Chen et al., 2020). The function serves to halt the enzymatic reactions in the fruits, which otherwise might cause loss of flavor, color and texture (Salau et al., 2015). The boiling or heating treatment in elderberry fruits was mainly to reduce the potential toxicity, although it can be expected that the process may deactivate some enzymes in elderberries as well. Based on the results of antioxidant activity assays (Table 7) and major secondary metabolite groups (Table 6), boiling extracted more total flavanols, total hydroxycinnamates and total anthocyanins and final EB wines showed higher antioxidant capacities.

Conclusions

Elderberry is well known for its medicinal functions, but little research has examined its fermented products. This study focused on the effect of different processing methods on elderberry wine properties and qualities. Two days of cold soak maceration (EC2) of elderberry musts have influenced its pH. The boiling treatment (EB) produced darker elderberry wine the darkest color based on L* values, whereas EC2 had the lightest color. Although the three treatments did not cause differences in ethanol content, glycerol content, total acidity, volatile acidity, or tannins, they did cause differences in wine pH levels. EC0 and EC2 had higher pH compared to EB-treated fermentation wine.

Examining monomeric phenolic compound concentrations, elderberry must treatments did not cause significant differences in total flavanols or total flavonols, but EB-treated fruit wine showed the highest anthocyanins concentration which corresponded to the darkest L* values in the color dynamic evaluation. Antioxidant activity assays and anthocyanin content indicated the EB treatment extracted the most antioxidants and anthocyanins. Cold soaking of elderberry fruit did not

improve the antioxidant potential in either must or wines for the EC2 treatments. Added heat or high-temperature treatment of elderberry fruit and must indicated that treatment length and processing influenced the extraction of specific compounds. It is worth further research on the use of extended soaking as well as specific maceration effects on elderberry fruit wine chemistry and perceived quality.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The work was supported by the MDA Montana Department of Agriculture) [AM22SCBPMT1127].

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