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Exploring Alternative Methods for Extracting Bioactive Phenolics from Winemaking Byproducts

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Exploring Alternative Methods for Extracting Bioactive Phenolics from Winemaking Byproducts

By

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Abstract

Wine grape pomace, composed of skins, seeds, stems, and pulp, is the major byproduct of the winemaking industry. While this byproduct can be diverted towards other agricultural uses, such as compost or animal feed, the environmental burden and poor animal digestibility of the material hinders the sustainability and effectiveness of these low-value mitigation strategies. Since wine grape pomace contains valuable bioactive compounds, especially phenolics, there remains an opportunity to valorize this material to benefit human health with the development of innovative food, beverage, cosmetic, and supplement applications. The extraction of wine grape pomace phenolics typically relies on the use of hazardous solvents like ethanol or methanol, which requires subsequent downstream processing steps for use in food-grade products. To present an eco-friendly alternative, this thesis focuses on the design of green extraction methods that improve phenolic extractability and support the *in vitro* antioxidant activity of grape pomace extracts while using water as the only solvent under optimized extraction conditions.

In Chapter 1, an overview of the recent advances and research gaps in green extraction technologies is discussed with a focus on the large-scale feasibility of these methods to support the potential for commercial adoption. In addition, the compositions of various winemaking byproduct fractions (pomace, leaves, lees, vinasse, and wastewater) are highlighted to showcase the diversity of components available for the development of value-added products.

In Chapter 2, the aqueous extraction process (AEP) is explored as an environmentally-friendly strategy for enabling the release of phenolics from the grape cell-matrix without the use of conventional solvents. A series of experiments, using a central composite rotatable design paired

with a kinetic study, was used to identify the concurrent effects of pH, solids-to-liquid ratio, temperature, and time on the total phenolic content (TPC) of the grape pomace extracts. To reduce water consumption without compromising phenolic extractability, a two-stage countercurrent method was employed, which recirculates the extraction processing fractions and increases the concentration gradient to drive phenolic diffusion. This technique provides an economically-viable and environmentally-friendly alternative to conventional solvent extraction methods.

The findings from Chapter 2 (i.e., the role of alkaline conditions in phenolic extraction) guided the design of enzyme- and microwave-assisted extractions as outlined in Chapter 3. Enzyme specificity and alkaline conditions enable the degradation of ester- and ether-linkages between phenolics, structural proteins, and cell-wall carbohydrates within the grape cell-matrix, while microwave radiation causes rapid temperature and pressure changes that can rupture the cell. Multiple stepwise screening experiments were performed to select the optimal conditions for maximizing TPC while reducing total enzyme concentration, water usage, and extraction time. The structural changes to the grape cell-matrix, as illustrated by scanning electron microscopy, represent the successful cellular disintegration caused by the synergistic effects of enzymatic hydrolysis, microwave heating, and intracellular pressure on promoting phenolic extractability.

The series of studies herein provides new approaches to extracting phenolics from wine grape pomace. Notably, the starting material used in these studies represents a non-conventional winemaking process using red wine grapes (*Vitis vinifera* L. cv. Cabernet Sauvignon) to produce white wine. The use of red wine grape pomace collected prior to fermentation ultimately affected

the phenolic composition of the grape pomace extracts, as detailed in Chapters 2 and 3 with the abundance of flavonol glycosides, which represent high residual sugars in the matrix. This finding emphasizes the influence of upstream winemaking conditions on the downstream phenolic profiles and potential biological properties of the extracts. In addition, this study offers support for the use of alkaline treatments for the extraction of phenolics from wine grape pomace, which is not yet widely reported due to the use of acidic conditions in conventional ethanol and methanol extractions. These insights, along with further work on scaling-up the microwave and enzyme-assisted extraction process, illuminate the possibilities for green extraction methods to revitalize agricultural byproducts while boosting human health, supporting a circular economy, and promoting environmental sustainability.

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Table of Contents

Research Communication

Oral Presentation

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Glossary

AAPH: 2,2'-Azobis(2-methylpropionamidine) dihydrochloride
ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AEP: Aqueous extraction process
BOD: Biological oxygen demand
CCRD: Central composite rotatable design
COD: Chemical oxygen demand
CSE: Conventional solvent extraction
EAE: Enzyme-assisted extraction
GAE: Gallic acid equivalent
HCl: Hydrochloric acid
ORAC: Oxygen radical absorbance capacity
MAE: Microwave-assisted extraction
MEAE: Microwave enzyme-assisted extraction
MSE: Microwave solvent extraction
NaOH: Sodium hydroxide
SLR: Solids-to-liquid ratio
TE: Trolox equivalent
TPC: Total phenolic content

Chapter 1: Green extraction technologies for the valorization of polyphenols from wine grape pomace: Recent advances and next steps for validating large-scale feasibility

1.1. Introduction

The valorization of agricultural byproducts plays a critical role in reducing the environmental impact of global food production. To manage the escalating climate crisis, the industrial production system must cooperate to design timely, affordable, and effective solutions for the agri-food supply chain by designing processes that limit water, energy, and land inputs while preventing, reducing, or reusing waste streams. In general, since all steps of the food supply chain have the potential for producing loss or waste, there is a need to establish mitigation strategies at all levels of production, with all varieties of crops, to transition to a more circular system where byproduct utilization is enhanced.

In 2020, the global vineyard surface area was estimated at 18 million acres with approximately 75% of the land dedicated to growing wine grapes (OIV, 2021), making this one of the world's most cultivated specialty crops. During grape processing for the production of wine, grape pomace is produced as a byproduct composed of seeds, skins, stems, and pulp remaining after the grape crush. Also known as marc, grape pomace residues represent approximately 20–30% of the total mass of pressed grapes (Dwyer et al., 2014). While this byproduct is typically diverted towards other agricultural uses such as compost, animal feed, or fertilizer, the high chemical oxygen demand (COD) of grape pomace presents environmental toxicity risks, and its poor digestibility limits the effectiveness of this waste stream as livestock feed. In some cases, grape pomace may even be sent to landfills or incinerated when the supply

outweighs the demand for these low-value diversion strategies, which further exacerbates the associated issues of air, land, and ground water pollution (Christ & Burritt, 2013).

Therefore, the underutilization of nutrients and valuable compounds from winemaking waste streams provides a multitude of opportunities for re-envisioning these byproducts for value-added applications. Winemaking waste streams can be valorized individually for niche applications requiring isolated components, such as grapeseed oil, or the material can be upcycled as a combination of multiple fractions to utilize as much of the byproduct as possible through the biorefinery process. A techno-economic analysis by Jin et al. (2021) determined that a full biorefinery scenario (i.e., production of grape seed oil, polyphenols, and biochar) provided an economically viable solution for addressing grape pomace with a 34.3% rate of return over a payback period of 2.5 years. Since multiple stable markets already exist for value-added products stemming from agricultural processes (e.g., ingredient markets in food, beverage, cosmetic, supplement, and packaging industries), further exploration of grape pomace valorization is warranted.

Grape pomace is rich in a wide variety of fatty acids, amino acids, and minerals as well as indigestible fiber, oligosaccharides, and phytonutrients, which have been associated with synergistic health benefits for the cardiovascular and gastrointestinal systems due to their antioxidant, anti-inflammatory, and antimicrobial properties (Bordiga et al., 2019; Saura-Calixto, 2011). The specific phytochemical profile of wine grape pomace can vary due to differences in wine grape cultivar, grape growing region and climate, soil type, winery scale, winemaking methods, drying and storage conditions, composition of pomace material (individual or mixed components: skins, seeds, stems, pulp), and extraction methods. For these reasons, it is challenging to directly compare data across studies analyzing grape pomace, but the support for

continued research efforts on sustainable and innovative processing approaches will help to broaden the understanding of the bioactive potential of the feedstock.

Conventional extraction technologies for obtaining valuable bioactive compounds from natural products typically rely on the heavy use of flammable or hazardous solvents that require costly and laborious clean-up operations for the development of food-grade products. To circumvent the human and environmental health concerns associated with toxic solvent use, greener extraction technologies have started to become more widely integrated into downstream processing systems with the growing support from global legislative authorities (United Nations, 2019).

Green extraction technologies that use benign solvents are therefore imperative for ensuring the environmental and economic sustainability of the agri-food chain while promoting substantial yields of phytochemicals, which often provide improved biological or functional properties (Ilyas et al., 2021; Moro et al., 2021; Pedras et al., 2020; Romulo, 2020). Examples of green techniques used to extract valuable compounds from winemaking byproducts include enzyme-assisted extraction, microwave-assisted extraction, ultrasound-assisted extraction, pulsed electric field extraction, pressurized hot water/subcritical water extraction, and supercritical fluid extraction. Each of these methods provides a unique chemical and physical mechanism for promoting the extraction of polyphenolics and other valuable components from the wine grape cell matrix while eliminating the dependence on harsh solvents (Figure 1.1).

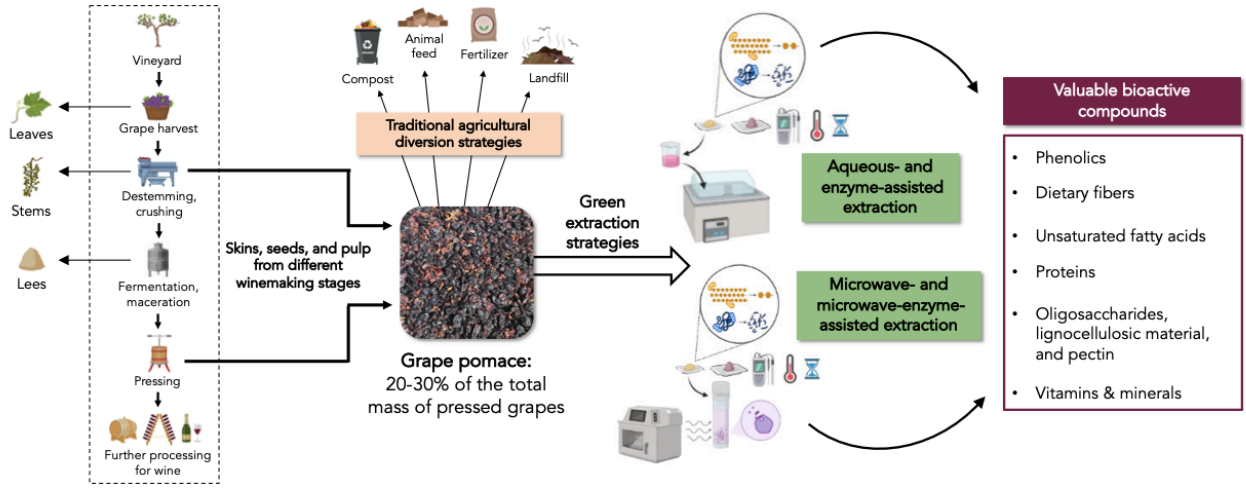

Figure 1.1. Pathways for the valorization of wine grape pomace. Winemaking byproducts can be converted into products for traditional agricultural applications or explored as a valuable bioactive feedstock using green extraction methods. Graphics created with BioRender.com, Freepik, and Flaticon.

The main objective of this review is to explore the recent accomplishments in state-of-the-art technologies for the extraction of phenolics from wine grape pomace. The latest bioprocessing strategies for revitalizing wine grape byproducts are highlighted, and the fundamental need to understand how the structural composition of these byproducts can be altered by the processing conditions is discussed. These insights aim to further guide the development of structure-function based processes for enhancing the extraction of compounds with the desired composition and functional/biological properties. The ample diversity of the many byproducts associated with winemaking will be explored to highlight the diversity of bioactive compounds available for potential food, beverage, biomedical, cosmetic, packaging, and nutraceutical applications. In addition, the various forms of phenolics (free, esterified, and insoluble-bound) will then be discussed to provide a mechanistic understanding of how phenolics are extracted from the grape cell matrix. In this review, conventional solvent extraction methods will be compared to alternative green techniques (specifically enzyme-assisted extraction,

microwave-assisted extraction, and the integration of these methods) to illuminate the potential of eco-friendly strategies to replace or complement traditional techniques for phenolic recovery. Recent advances and challenges in scaling these extractions to promote feasible, large-scale commercialization efforts for green extraction of winemaking waste streams will also be discussed.

1.2. Winemaking byproduct fractions and their compositions

Although the largest solid mass fraction of winemaking waste streams comes from the skins, seeds, stems, and pulp (collectively known as pomace or marc), a multitude of other byproducts are generated during processing that should also be considered for their valuable biological and functional properties. For example, leaves are removed during grape harvesting, lees remain after fermentation as a sediment of yeast cells, vinasse is generated as a liquid fraction after the distillation process, and wastewater is produced as an effluent throughout production and cleaning (Gómez-Brandón et al., 2019). Each of these fractions provides an opportunity to recover valuable components that can be further manipulated for its bioactivity through targeted downstream processing and formulation. Continued research on developing cost-effective and eco-friendly methods for managing winery waste streams is needed to expand the portfolio of valuable products associated with these effluents and will ultimately establish feasible, closed-loop industrial systems.

1.2.1. Grape pomace or marc: a combination of skins, seeds, stems, and pulp

It is estimated that a typical winery using 9–13 tons of freshly crushed wine grapes will produce about one ton of grape pomace, which is composed of approximately 50% skins with low amounts of residual pulp, 25% seeds, and 25% stems (Spinei & Oroian, 2021). While grape pomace can contain about 50–70% water (Kyzas et al., 2014), the solid residues provide valuable

compounds including polyphenols, fiber, fatty acids, vitamin C, iron, and pectin (Castro Sousa et al., 2014).

Grape skins contain approximately 5–12% protein, 1–6% fat, and 3–8% ash depending on the variety, climate, and growing conditions of the grapes (Deng et al., 2011). In red grape varieties, anthocyanins like malvidin, cyanidin, petunidin, delphinidin, and peonidin glycosides provide a deep color to finished red wine products. These compounds also provide health benefits including quenching of free radicals and inhibiting oxidation of low-density lipoprotein (LDL) (Yildirim et al., 2005). White wine grapes, like Chardonnay, are reported to contain higher contents of catechin (60 mg/100 g dry matter) and epicatechin (44 mg/100 g dry matter) compared to Merlot grapes (16 and 13 mg/g100 g dry matter, respectively) (Yilmaz & Toledo, 2003). Since white wine grape skins are obtained after pressing and thus separated from fermentation, they also contain higher soluble sugar contents with reports of 55.8% soluble sugars in Muller Thurgau skins and 77.55 in Morio Muscat skins (Deng et al., 2011). Conversely, red wine grape skins from Cabernet Sauvignon, Merlot, and Pinot Noir varieties remain in contact with the juice during fermentation and thus have low soluble sugars (1.34–1.71%) and high total dietary fiber (51.1–56.3%) (Deng et al., 2011).

Grape seeds compose 3–6% of the total weight of the grape and provide the main source of monomeric tannins in the grape pomace, which are typically associated with bitter and astringent notes in wines. Wine grape seeds are rich in other flavan-3-ols such as catechin (approximately 50% of the total polyphenolic content), epicatechin (26%), epicatechin gallate (9%), and procyanidins B1 and B2 (11%) with lower levels of epigallocatechin and gallic acid (Guendez et al., 2005). Grape seeds are valuable for their radical scavenging activity provided by procyanidin B1, but they are also exploited for their high content of unsaturated fatty acids that

are beneficial for the high smoke-point (268 °C) of grapeseed oil (Alzaa, 2018). Linoleic acid (omega-6), linolenic acid (omega-3), oleic acid, and palmitic acid are most prevalent in grape seeds, providing health benefits towards preventing cardiovascular disease, hypertension, and autoimmune disorders as well as offering wound-healing properties (Gupta et al., 2020). Additionally, grapeseed oil contains vitamin E and phytosterols like stigmasterol and β-sitosterol (Shinagawa et al., 2015). Grape seeds also contain approximately 35% fiber, providing an even richer dietary fiber source than the skins.

Depending on the grape variety, vintage, and maturity, grape stems are composed of 12–38% cellulose, 10–21% hemicellulose, 13–47% lignin, 5-11% protein, and low amounts of ash, sugars, and organic acids (Blackford et al., 2021). The incorporation of grape stems in the winemaking process depends on the style of wine produced and the desired sensory properties of the finished wine. In white wines, stems sometimes remain attached to the grape to aid in juice extraction, while in red wines, stems are typically removed before maceration and pressing to prevent excessive astringency and "green" notes from the presence of hexanal in the stems (Hashizume & Samuta, 1997). The total polyphenol concentration in stems from red wine grapes ranges between 400 to 22,900 mg GAE/g dry matter and from 348 to 38,400 mg GAE/g dry matter in white wine grape varieties (Blackford et al., 2021). The major phenolic acids in red and white grapes are caftaric, coutaric, and gallic acid with reportedly high contents in Malvasia Fina (Leal et al., 2020) and Cabernet Sauvignon stems (Kosińska-Cagnazzo et al., 2020). Other polyphenols like quercetin-3-O-rutinoside, astilbin, engeletin, catechin, epicatechin, trans-resveratrol, and ε-viniferin have also been identified in stems, which carry potent antioxidant activities.

Residual grape pulp attached to the grape skins contains low levels of phenolics since the pulp is mainly composed of pectic polysaccharides. In the grape cell wall, the middle lamella layer contains the major fraction of pectic substances in the form of homogalacturonan and rhamnogalacturonan I and II (Gao et al., 2019). Other polysaccharides like arabinogalactoproteins, arabinans, and homorhamnogalacturonans also exist in grape cell walls (Barnavon et al., 2000). Pectin can be recovered from grape pomace using acidic ultrasound-assisted extraction (Minjares-Fuentes et al., 2014) and provides functional properties as gelling and thickening agents in food and beverage, cosmetic, pharmaceutical, and supplement applications.

1.2.2. Leaves

Grape leaves are a byproduct of pruning and harvesting. Traditionally, grape leaves can be preserved in brine and are used in a variety of cuisines, but they also have associated health benefits for the treatment of hypertension, high blood sugar levels, and inflammation. Dani et al. (2010) reported that *Vitis labrusca* (Bordo) grape leaf extracts from both conventional and organic grapevines contained high contents of rutin (45–55 mg/g extract) as well as lower levels of quercetin, catechin, kaempferol, and resveratrol. These extracts were successful in reducing protein and lipid damages induced by hydrogen peroxide to mediate oxidative stress conditions in animal models (Dani et al., 2010). Phenolic compounds that were unique to wine grape leaves compared to the finished wine, grape stems, pulp, and skins include chlorogenic acid hexoside, dihydroxybenzoic acid hexosyl pentoside isomers, and ellagic acid pentoside (Šuković et al., 2020).

1.2.3. Lees

Wine lees is the acidic sediment of yeast cells and other precipitates (e.g., tartaric acid) remaining after the alcoholic fermentation and filtration stages of the winemaking process. This fraction is rich in residual polyphenols like other winemaking fractions, including phenolic acids, flavonols, and anthocyanins, but it also provides unique attributes from mannoproteins (i.e., mannose residues linked to polypeptide chains) and β-glucans from the yeast cell wall (Pérez-Serradilla & de Castro, 2008). Mannoproteins can interact with polyphenols through adsorption, which has been shown to improve color stability and reduce the astringency of wines (Vidal et al., 2004). Wine lees mannoproteins also function as wine stabilizers to prevent protein and tartrate precipitation (Ribeiro et al., 2014).

1.2.4. Vinasse

Vinasse is the acidic effluent remaining after grape pomace has been distilled for the extraction of ethanol and tartaric acid. It is estimated that 1 L of ethanol produced through distillation generates 10–15 times the volume of vinasse (Tena et al., 2020). The biological and chemical oxygen demand (BOD and COD, respectively) of vinasse has measured 10,440 mg/L and 23,304 mg/L with high values of total organic carbon (9710 mg/L) and nitrogen (231 mg/L) (Barbanera et al., 2021). Innovative treatment efforts of winery vinasse include the conversion of the high-moisture waste stream to hydrochar by hydrothermal carbonization, which is a novel process that converts vinasse into a solid coal-like renewable energy source and an enriched aqueous co-product without the need for dewatering unit operations (Barbanera et al., 2021).

1.2.5. Wastewater

Winery wastewater is mainly a byproduct of cleaning operations and therefore primarily contains cleaning agents like caustic solutions, organic residues, and salts. Like vinasse, winery

wastewater has been reported to generate high COD (> 15,000 mg/L on average) due to the presence of suspended solids, organic acids, fatty acids, polyphenols, and low amounts of chemical fertilizers, pesticides, and herbicides that are commonly applied to grapes in the vineyard (Kyzas et al., 2014; Mosse et al., 2011). Inorganic components including moderate concentrations of sodium, potassium, calcium, magnesium copper, zinc, and lead also contribute to the ecological impact of winery effluents. The high COD of this waste stream, therefore, necessitates robust water treatment efforts to reduce the concentration of pollutants in the water system. Standard methods and emerging techniques for recovering chemical components from winery effluents include physical settling methods, biological treatments using bacteria, and chemical treatments like electrodialysis or ion exchange (Bharathiraja et al., 2020). Recovered components, like sugars and organic matter, from the waste streams have been used as starting materials for biofuels and bio-oils (Ganeshkumar et al., 2018) and as substrates for enzyme production (especially Aspergillus sp.) (Salgado et al., 2016).

1.3. Free, esterified, and bound forms of polyphenols in the grape cell matrix

While numerous valuable components are available for recovery from byproducts produced throughout the entire winemaking process, as outlined in Section 1.2, the pomace fraction is most notable due to the sheer volume produced with each harvest and the potential for phenolic recovery. To better understand the types of methods, and the respective challenges, for extracting phenolics from wine grape pomace, the forms by which phenolics are present in the cell wall must be first elucidated to establish the mechanism of action associated with their recoveries.

Polyphenols are present in free, esterified, and insoluble-bound forms within plant cells (Figure 1.2). Free phenolics are readily solubilized in solvents such as water, ethanol, methanol,

and acetone, and esterified phenolics linked to low-molecular-weight compounds like short-chain fatty acids or soluble sugars are also recoverable by organic solvents (Yu et al., 2019). These soluble phenolics (both free and esterified forms) exist within the plant cell cytoplasm or inside the cell vacuole, which become available for extraction after the cell is ruptured by chemical, enzymatic, or physical degradation. Most reports on polyphenolic contents in natural products target free or readily extractable phenolics with the use of aqueous organic solvents. Examples of free and esterified phenolics that have been identified in wine grape byproducts include isoquercitrin, resveratrol, rutin, ellagic acid, ferulic acid, caftaric acid, kaempferol hexoside, quercetin hexoside, myricetin hexoside, and procyanidin trimers (de Camargo et al., 2014; Spinei & Oroian, 2021).

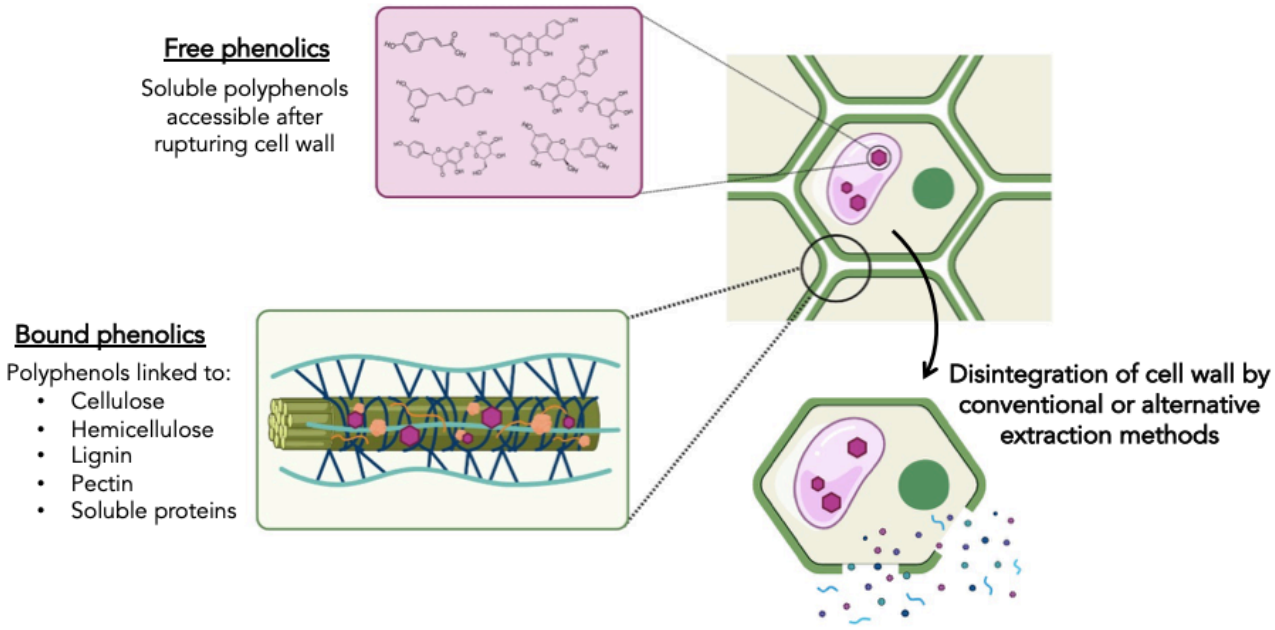

Figure 1.2. Schematic of soluble free phenolics within a plant cell vacuole and insoluble-bound phenolics within a plant cell wall. Conventional or alternative green extraction methods are used to rupture the plant cell wall for phenolic recovery. Graphics created with BioRender.com.

On the other hand, insoluble-bound phenolics require more intense processing for their extraction from plant cell matrices due to covalent linkages that bind these compounds to structural material and form protein-polyphenol and polysaccharide-polyphenol conjugates. “Non-extractable” or bound phenolics can be chemically linked to cell wall components like cellulose, hemicellulose, lignin, pectin, or soluble proteins (Figure 1.2) through ester and ether linkages, hydrophobic interactions, and hydrogen bonds. Common strategies that can enable the release of insoluble-bound phenolics from the plant cell matrix include acid and alkaline hydrolysis, enzymatic hydrolysis, fermentation, and thermal treatments like roasting, boiling, and extrusion cooking (Shahidi & Yeo, 2016). It is important to consider incorporating these extraction strategies that impart additional pressure, temperature, and/or pH changes in the cell matrix because it is estimated that insoluble-bound phenolics represent a larger portion of the total phenolic profile in plant foods than free or esterified compounds (Arranz et al., 2009). In a previous study, de Camargo et al. (2014) reported that insoluble-bound phenolics composed approximately 80% of Syrah and Tempranillo byproducts while the esterified and soluble phenolics composed only 20% of the profile. In the same study, procyanidin dimer A was found only in the insoluble-bound form in the winemaking byproducts, while substantial contents of procyanidin dimer B, protocatechuic acid, p-coumaric acid, gallic acid, caffeic acid, ellagic acid, catechin, and prodelphinin A and B were also reported in the bound phenolic fraction (de Camargo et al., 2014).

Bound polyphenols provide additional health benefits beyond the commonly associated antioxidant properties due to their unique structure-function characteristics. Since these phenolics are linked to insoluble compounds like cellulose, hemicellulose, structural proteins, and pectin, they reach the colon largely still intact without being absorbed by the small intestine

(Andreasen et al., 2001). In fact, only about 5–10% of ingested phenolics are absorbed in the small intestine while the remaining 90–95% enter the colon. It is evident that designing extraction strategies to recover all three forms of phenolics (free, esterified, and insoluble-bound) in tandem would help address the current knowledge gaps associated with understanding the abundance, diversity, and functionality of grape pomace bioactives.

1.4. Techniques for extracting polyphenols from wine grape pomace

As discussed in previous sections, polyphenols from wine grape pomace have traditionally been extracted using aqueous organic solvents with polar affinities. Although effective, the use of hazardous or flammable solvents in these strategies increases human health and safety risks as well as environmental concerns associated with their disposal. In this section, an overview of both conventional solvent extraction methods and recent advances in green technologies for extracting polyphenols from grape pomace will be discussed. Specifically, the mechanisms of action associated with the extraction of phenolics using alternative strategies such as enzyme-assisted, microwave-assisted, and integrated methods will be discussed.

1.4.1. Conventional solvent methods for phenolic extraction

Solid-liquid extraction of polyphenols from wine grape pomace typically relies on sample fractionation using a range of solvents with varying degrees of polarities. Table 1.1 highlights a variety of solid-liquid extraction methods using both red and white grape materials extracted with a variety of solvents, extraction times (5–1440 min), and temperatures (20–60 °C). Extractions using ethanol, ethyl acetate, butanol, dichloromethane, and n-hexane were used to fractionate Cabernet Sauvignon grape pomace based on the descending polarity of the solvents (de Campos et al., 2008), but this traditional Soxhlet method (the “gold standard” for bioactive extraction from plant material) required 6 h of extraction under continuous reflux. While the

extended period of contact between grape material and solvent should increase the mass transfer of solutes, the extensive extraction time requires excessive volumes of flammable or otherwise hazardous solvents. In another study, Pintać et al. (2018) concluded that 80% methanol is the best solvent for maximizing phenolic extraction efficiency at large scale, regardless of its purity, but suggested using ethyl acetate for increasing polyphenolic richness, acidified 50% methanol for anthocyanin isolation, and acetone for triterpenoid (e.g., ursolic acid) extraction. However, there is a risk of thermal degradation of phenolics when using these solvents at temperatures at their boiling points (as low as 56 °C for acetone) for long periods of time.

Table 1.1. Conventional solvent extraction methods and phenolic yields from wine grape pomace

Grape variety	Sample preparation	Extraction parameters				Total phenolic content (TPC)	Reference
		Solvent	Solids-to-liquid ratio (SLR)	Time (min)	Temp. (°C)		
White and red Garnacha skins, stems, and seeds	Grapes were pressed or distilled. Dried and stored at room temperature	Methanol, 96% ethanol, and water	1:1-1:5	30-90	25-50	16-163 mg GAE/g dry residue	Pinelo et al., 2005
Albariño white pomace collected after distillation	Frozen at -50 °C. Air-dried and crushed to particle size 0.6-1 mm	Ethanol	27 g pomace with flow rate of 2-4 mL solvent/min	100	40-50	0.032-0.065 mg GAE/100 g dry pomace	Guerrero et al., 2008
Red grape pomace	Dried at 60 °C for 24 h. Skins ground to particle size of 270.8 ± 26.4 μm	Ethanol (8-92%)	1:3-1:17	60	30	5.7-48.6 mg GAE/g dry grape skin	Caldas et al., 2018
Cabernet Sauvignon, Italian Riesling, and Merlot pomaces	Fresh samples	80% methanol, 80% ethanol, acetone, ethyl acetate, and methanol:distilled water:formic acid as 50:48.5:1.5 and 80:19:1	1:10	360	ambient	16.9-110 mg GAE/g dry extract or 0.28-7.77 mg GAE/g fresh weight	Pintać et al., 2018
Isabel (*Vitis labrusca*) processing residue	Oven-dried at 40 °C for 18 h. Ground in a cooled knife mill to a particle size of 355-180 μm. Vacuum packed and stored at -18 °C	Ethanol acidified with 1.5 mol/L citric acid solution (80:20 v/v)	1:15-1:25	20-80	20-60	6.23-8.80 mg GAE/g dry sample	Chañi-Paucar et al., 2020
Corvina and Corvinone red wine pomace collected after pressing, maceration, and fermentation	Dried at 40 °C for 24 h. Ground to particle size < 500 μm. Treated with nitrogen at 90 °C for 90 min. Stored at room temperature for 0, 4, 9 months	Methanol or ethanol at 25, 50, and 75%	1:10	45	50	15-36 mg GAE/g dry sample	Cisneros-Yupanqui et al., 2020
Cabernet Sauvignon pomace	Dried at 40 °C for 24 h up to 9.8 ± 0.1% moisture. Ground to a particle size of 0.91 mm	Ethanol, ethyl acetate, butanol, dichloromethane, *n*-hexane	5 g sample with continuous reflux (Soxhlet)	360	ambient	1.3-3.4 g GAE/mg extract	de Campos et al., 2008
Barbera pomace	Oven-dried at 60 °C to 2-4% moisture content. Milled to particle size ≤ 2 mm	Ethanol:water (60:40 v/v)	1:4	5, 15, 30, 60, 120, 180, 240, 300	60	6.0-8.5 g GAE/L extract	Amendola et al., 2010
Red grape marc	Dried to 14.3% moisture and ground to a particle size of 0.83 mm	Ethanol:water (40:60, 65:35, and 90:10 v/v)	1:10, 1:30, 1:50	360, 900, 1440	ambient	7.89-23.36 mg GAE/g dry matter	da Porto and Natolino, 2018

1.4.2. Alternative green extraction methods

Compared to traditional solid-liquid extractions, enzyme-assisted (EAE) and microwave-assisted (MAE) extractions provide opportunities for reduced solvent consumption, reduced extraction time, and improved biological or functional activity of the extracts (Figure 1.3). While the chemical behavior of ethanol or methanol can be advantageous for polyphenolic extractions, the high polarity and dielectric constant of water are especially important factors in green techniques because these characteristics offer excellent solvent specificity, allowing for the use of milder conditions, and providing efficient absorption of microwave radiation. Additionally, EAE can be optimized alongside other extraction parameters to reduce total enzyme loading for optimal economic feasibility, and MAE can shorten overall extraction times for the development of high throughput processing systems.

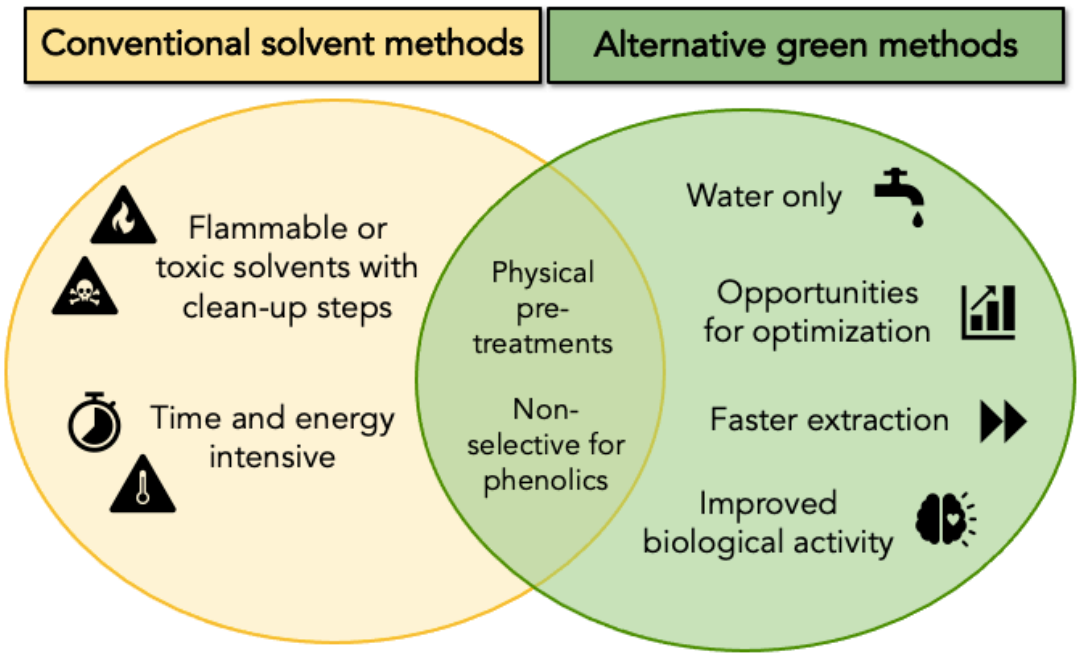

Figure 1.3. Comparison of conventional and alternative green techniques for phenolic extraction

1.4.2.1 Enzyme-extraction process (EAE)

The addition of enzymes to aqueous extractions aids in catalyzing the hydrolysis of plant cell-wall proteins and polysaccharides, which can help release insoluble-bound phenolics from the cell matrix. Enzyme preparations commonly used in extracting phenolics from wine grape pomace include cellulase, hemicellulose, glucanase, pectinase, xylanase, tannase, and protease (Table 1.2). Since enzymes are easily degraded in harsh environments, the control of extraction parameters like pH and temperature is especially critical for promoting optimum enzymatic activity. Additionally, the extraction time, solvent type and concentration, and enzyme type and dosage also impact enzyme extraction kinetics, extraction efficiency, and economic viability of the process.

Many studies in Table 1.2 reported that the use of multiple enzymes at various dosages best induced polyphenolic extraction from wine grape pomace samples (Chamorro et al., 2012; Martins et al., 2016; Wang et al., 2019). The addition of 3% (w/w) Viscozyme® L, an enzyme complex including arabinase, cellulase, β-glucanase, hemicellulase and xylanase activities, increased the total phenolic content of Croatina red grape skin powders by 56% compared to solvent extraction using 60% (v/v) ethanol (Binaschi et al., 2018). While the high enzyme loading and extraction time of this study (120 min) could be improved through further optimization experiments, this example provides insight into the potential for EAE to improve phenolic yields without reliance on flammable solvents.

Notably, nearly all the studies listed in Table 1.2 used a sodium acetate buffer solution to mediate pH fluctuations during the enzymatic extractions, emphasizing the role of pH in enhancing biochemical activity and phenolic recovery. The pH of the extracting solvent in these

studies ranged from 2.0 to 6.0, but further research evaluating alkaline conditions to release insoluble-bound phenolics is warranted and will be explored in Chapter 2.

Table 1.2. Enzyme-assisted extraction methods and phenolic yields from grape pomace

Grape variety	Sample preparation	Extraction parameters						Total phenolic content (TPC)	Reference
		Solvent	Solids-to-liquid ratio (SLR)	Enzyme(s) and concentration(s)	pH	Time (min)	Temp. (°C)		
Red wine grape pomace	Dried at 60 °C and milled to 1 mm	0.1 M sodium acetate buffer	1:10	Pectinase (6.75-13.5 U/g pomace), cellulase (157.5-315 U/g pomace), and tannase (500-1000 U/g pomace)	5.5	1440	35	1.0-1.4 mg/g dry pomace	Chamorro et al., 2012
Merlot, Cabernet Sauvignon, and Cabernet Franc pomace	Dried to moisture content of 10–11%. Milled to pass a 40-mesh screen	0.1 M sodium acetate buffer	1:6	Pectinase (20 U/g pomace) and cellulase (315 U/g pomace)	4.5	1440	50	2.93-8.40 mg GAE/g dry matter	Wang et al., 2019
Syrah and Seibel hybrid, Moscato branco, and mixed red and white grape pomace	Dried, ground, and stored at -80 °C	0.02 M sodium acetate buffer	1:100	Tannase, pectinase, cellulase at 5% w/w of tannase; 2.5% of pectinase and cellulose each; and 1.66% of tannase, pectinase, and cellulase each	5.0	300	40	19.3-82.1 mg GAE/g dry matter	Martins et al., 2016
Syrah, Cabernet Sauvignon, Malbec, Pinot-Noir, and Marselan pomace	Dried to < 6% moisture and ground to 0.25-2.38 mm	50 mM acetate buffer	1:10	Pectinase (0-100 U/g pomace), cellulase (0-100 U/g pomace), and tannase (0-200 U/g pomace)	4.0-5.5	120-360	25-45	3.9-8.6 mg GAE/g pomace	Meini et al., 2019
Cabernet Sauvignon and Merlot	Skins removed, lypophilized, and ground to 125–250 µm	0.2 M acetate buffer	1:10	Cellulase, pectinase, and polygalacturonase at 1-10% enzyme/substrate loading	3.6	120-360	25-55	18-55 mg GAE/g dry grape skin	Arnous & Meyer 2010
Wine grape solid waste	Dried at 60 °C for 24 h, then ground to 30 mesh particle size	0.2 M acetate buffer	1:1.4	Cellulase, pectinase, and β-glucosidase diluted 1:10	3.5	60-2880	40	0.27-0.40 mg GAE/g dried grape waste	Gomez-Garcia et al., 2012
Alicante Bouschet pomace	n.a.	0.02 M sodium acetate buffer at pH 5.0	1:8	Enzyme preparation with polygalacturonase, carboxymethylcellulase, and β-glucosidase activities	5.0	5-30	32	4-9 mg GAE/g dry poamce	Cascaes Teles et al., 2021
Regent seeds	Dried and ground to fine powder	Water with 0.015 M calcium ions	1:80	Enzyme preparations with polygalacturonase, pectinase, cellulase, and hemicellulase activities	2.0-4.0	60-180	40-50	0.11-0.24 mg GAE/g dry weight	Štambuk et al., 2016
Sangiovese and Merlot skins	Dried at 60 °C for 24 h. Milled to particle size 0.25-2 mm	Water	1:7, 1:24	Enzyme preparation with arabinase, cellulase, β-glucanase, hemicellulase and xylanase activity (3% w/w)	6.0	120	24	15.27-31.60 mg GAE/g dry weight grape	Binaschi et al., 2018

1.4.2.2. Microwave-assisted extraction (MAE)

In MAE, absorption of the microwave electromagnetic field (300 MHz to 300 GHz) by polar solvents ultimately induces degradation of the plant cell structure to aid in phenolic extraction (Žlabur et al., 2018). Dipole rotation of the polar solvent molecules produces heat as the water molecules oscillate to align with the field and generate friction. While the temperature of the intracellular matrix increases, the pressure also builds within the cell and weakens the cell wall, which eventually allows the solvent to penetrate the cell for recovery of secondary metabolites.

MAE is an advantageous green extraction method due to its simplicity of use. Interestingly, since the extraction mechanism of MAE relies on producing heat by oscillating polar molecules, the in-situ water content within plant cells can act as its own extraction solvent, thus eliminating or reducing the need for additional solvent consumption. Using only pressed dark-skinned grapes in a microwave system, Bittar et al. (2013) successfully produced grape extracts with a total phenolic content of 21.41 mg GAE/g dry weight. Microwave radiation therefore reduces the reliance on hazardous solvents while improving solvent permeation with increased temperature and reducing processing time. The extraction parameters of MAE can also be controlled for a more targeted reaction; variables include solvent type, solids-to-liquids ratio, sample particle size, moisture content and type of drying of sample, microwave power, extraction time, number of radiation cycles, vessel shape and size, vessel pressure, and extraction temperature (Kala et al., 2016). The design of the microwave vessel in an enclosed cavity aids in protecting light-sensitive compounds from oxidation, even though the short processing time (often less than 20 min, Table 1.3) can also help prevent sample degradation.

Table 1.3. Microwave-assisted extraction methods and phenolic yields from grape pomace

Grape variety	Sample preparation	Extraction parameters					Total phenolic content (TPC)*	Reference
		Solvent	Solids-to-liquid ratio (SLR)	Time (min)	Temp. (°C)	Power (W)		
Alphonse Lavallée grapes	Pressed at 200 bar	Solvent free (relying on water within grape cells)	n.a.	20	100	1 W/g press cake in 10 W increments up to 900 W	21.41 mg GAE/g dry weight	Bittar et al., 2013
Bordo and Isabel grapes	Crushed and heated 85 ± 1°C for 10 s, then stored at 4 °C	Hexane, ethanol, chloroform, ethyl acetate, ultra-pure water	1:5	20	110	850	0.13-1.07 mg GAE/g sample	Pezzini et al., 2019
Napoleon skins and seeds	Freeze-dried and ground	Methanol, ethanol, acetone, water	1:10-1:50	5-20	100	100-500	22.37 mg caffeic acid equivalents/kg	Azaroual et al., 2021
Tintilla de Rota grape skins	Ground and stored at -20 °C	50-80% methanol in water	1:12.5-1:25	5-20	50-100	100-500	0.0277-0.953 mg/g anthocyanins	Liazid et al., 2011
Tempranillo marc	*n.r.*	50:50 v/v ethanol:acidified water (pH 1 with sulfuric acid)	*n.r.*	1	80	300	45.9 mg GAE/g extract	Matos et al., 2019
South African Ribier seeds	Freeze-dried to 46% moisture and ground, then defatted	Methanol:water (90:10 v/v)	1:15	1-3.33	60-68	30-300	369-407 mg tannic acid equivalent/g extract	Hong et al., 2001
Chardonnay, Sauvignon Blanc, Cabernet Sauvignon, Shiraz seeds	Milled to fine powder	Ethanol (10, 30, 50, 70, 90%) in water	1:10-1:30	540-1740	25	100-200	47.2-86.6 mg GA/g grape seed	Li et al., 2011
Riesling marc seeds	Dried to a moisture content of 10-11% and milled to 80-300 µm	24-34% (w/w) acetone and 14-22% (w/w) ammonium citrate	*n.r.*	0.25-1.5	56	650	49.9-82.7 mg GAE/g marc (dry weight)	Dang et al., 2014
Red and white grape marcs	Air-dried and stored at room temperature	Water/acetone/acetic acid (29.5/70/0.5); addition of sodium carbonate (0 - 2.5%)	1:8	8	100	*n.r.*	1-16 mg GAE/g dry matter	Brahim et al., 2014
Bangalore blue seeds	Lyophilized, ground, and defatted	Ethanol (30-60%)	1:100	2-6	*n.r.*	100-200	9.138-15.241 mg GAE/g dry weight	Krishnaswamy et al., 2013
Agiorgitiko pomace	Stored at -30°C. Air dried or accelerated solar dried. Milled.	Water or water/ethanol (1:1 v/v)	1:50	60	50	200	24.05-231.61 mg GAE/g dry extract	Drosou et al., 2015
Chardonnay pomace	Dried 60 °C for 24h. Skins separated and milled to < 2mm	60% ethanol	1:04	0.825-1.83	46.5-74	100-900	6.68-9.96 mg GAE/g dry pomace	Pedroza et al., 2015
Tempranillo pomace	Stored at -18 °C and thawed at 4 °C overnight before extraction	50% ethanol and acid water (sulfuric acid, pH 1)	1:1.33	0.5-3	60-120	300	115.5-241.6 mg GAE/g dry extract	Álvarez et al., 2017
Chardonnay pomace	Stored at -18 °C. Defrosted at room temperature. Blended 1 min	30, 45, and 60% ethanol	1:5-1:10	5-15	24	93	0.322-1.315 mg GAE/mL extract	Garrido et al., 2019

*reported as gallic acid equivalents unless otherwise noted. *n.r.:* not reported

1.4.2.3. Integrated green extraction methods

The use of consecutive alternative extraction methods has shown synergistic effects on extracting phenolics from plant samples in recent studies. Techniques like EAE and MAE can be combined with each other or with other alternative extraction methods like ultrasound-assisted extraction to increase extraction efficiency while still consuming less energy than traditional Soxhlet methods (Barrera Vázquez et al., 2014). In one example, Wang et al. (2019) reported that microwave and ultrasonic-assisted consecutive extraction (MUAE) promoted the highest extraction yields and antioxidant activities across Merlot, Cabernet Sauvignon, and Cabernet Franc pomace, skin, and seed fractions compared to the use of these two strategies individually. Other novel applications of non-conventional phenolic extraction methods include enzyme and microwave co-assisted salting-out extraction (Jia et al., 2021), pressurized liquid extraction followed by integrated membrane filtration (Pereira et al., 2020), and the use of cyclodextrin extraction to assist ultrasound extraction (Alibante et al., 2021). In the latter example, the amphiphilic nature of β-cyclodextrin molecules, which are characterized by their external hydrophilic surface and the internal hydrophobic center, promoted the recovery and encapsulation of plant-derived compounds with varying degrees of polarities.

Despite the innovative approaches of the integrated strategies shown in Table 1.4, the use of ethanol as the extracting solvent is a persistent trend. In Chapter 3, an integrated microwave and enzyme-assisted extraction process will be designed using water as the only solvent under alkaline conditions. As a proof-of-concept experiment, this study will showcase the role of slurry pH while maximizing phenolic extractability with the addition of alkaline protease, which has not yet been extensively explored for its hydrolytic effects in grape pomace applications.

Table 1.4. Integrated green extraction methods and phenolic yields from grape pomace

Grape variety	Sample preparation	Green extraction methods	Extraction parameters									Total phenolic content (TPC)	Reference
			Solvent	Solids-to-liquid ratio (SLR)	Additional solids and loading	pH	Time (min)	Temp. (°C)	Power (W)	Amplitude (%)	Filtration system		
Merlot, Cabernet Sauvignon, and Cabernet Franc pomace	Dried to moisture content of 10–11%. Milled to pass a 40-mesh screen	Microwave-assisted extraction (MAE) + ultrasound-assisted extraction (UAE)	Ethanol/water (70:30 v/v)	1:20	*n.a.*	*n.a.*	4 min for MAE + 56 min for UAE	85-90 °C for MAE + 60 °C for UAE	800 W for MAE + 150 W for UAE	*n.a.*	*n.a.*	4.05-11.02 mg GAE/g dry matter	Wang et al., 2019
Agiorgitiko pomace	Dried to 8% moisture and milled to 0.3 mm	Microwave and enzyme-assisted extraction (MAE + EAE)	Ethanol, 42% v/v	MAE: 1:24	EAE: Cellulase pre-treatment, 4 % w/w, 240 min; 1:2 SLR	*n.a.*	EAE: 130 min	*n.a.*	MAE: 408	*n.a.*	*n.a.*	45.35 mg GAE/g dry pomace	Drevelegka and Goula, 2020
Agiorgitiko pomace	Dried to 8% moisture and milled to 0.3 mm	Ultrasound and enzyme-assisted extraction (UAE + EAE)	Ethanol, 53% v/v	UAE: 1:8	EAE: Cellulase pre-treatment, 4 % w/w, 240 min; 1:2 SLR	*n.a.*	EAE: 240 min	56	UAE: 130	34	*n.a.*	48.76 GAE/g dry pomace	Drevelegka and Goula, 2020
Cabernet Sauvignon pomace	Dried to moisture content of 7.83% and ground into powder to pass 120-mesh screen	Enzyme and microwave co-assisted salting-out extraction	Dipotassium hydrogen phosphate-citric buffer solution + 20% (w/w) ammonium sulfate and 25% (w/w) ethanol	1:12.5	Pectinase (450 U/g)	4.5	1.5 min for microwave + 60 min for salting-out	90 °C for microwave + 25 °C for salting out	270	*n.a.*	*n.a.*	125.32 mg GAE/g extract	Jia et al., 2021
Syrah marc	Ground and stored at -18 °C	Pressurized liquid extraction (PLE) + microfiltration and nanofiltration system (FS)	Ethanol/water (50/50%)	1 kg marc to 217 kg solvent	*n.a.*	2.0	15 extractions at 54 min each	40 °C for PLE and 35 °C for FS	*n.a.*	*n.a.*	MV020-NP010: 4 MPa, 1000-1200 g/mol pore size, 2.1 L feed volume	20.7 mg GAE/g dry weight with 71% phenolic retention using FS	Pereira et al., 2020
Muscat grape pomace	Dried at 80 °C for 450 min and milled to 0.384 mm	Ultrasound-assisted pretreatment (UAE) + β-cyclodextrin extraction (CD)	Water with 1 g/L citric acid	1:10-1:90	0-1.5% w/w CD	2.0	0-20 min for UAE + 180 min for CD	27-80	550 for UAE	*n.a.*	*n.a.*	16.67-57.47 mg GAE/g dry matter	Albiante et al., 2021

n.a.: parameter is not applicable to this study

1.5. Scaling up green extraction of grape pomace phenolics to produce value-added products

While there is an abundance of published literature on the success of green extraction methods for valorizing wine grape pomace at lab-scale, determining the effectiveness of these techniques at a larger scale is still an emerging field that requires further development of process optimization and process economics. Based on the results from small-scale experiments, phenolic extracts from winemaking waste streams have successfully served as bioactive ingredients in functional foods like bread, dairy, cookies, meat, pasta, and salad dressing (Coe & Ryan, 2016; Karnopp et al., 2015; Marinelli et al., 2015; Özvural & Vural, 2011; Santos et al., 2017; Tseng & Zhao, 2013); cosmetics like gels, creams, and sunscreens (Emmulo et al., 2021; Hübner et al., 2020; Surini et al., 2018); packaging materials (Cejudo-Bastante et al., 2021;

Coelho et al., 2020; Ferri et al., 2020; Saurabh et al., 2018), and supplements (Manca et al., 2020; Martínez-Maqueda et al., 2018; Ramos-Romero et al., 2021).

Despite the diversity of potential industrial applications of grape pomace, there remain opportunities to improve the environmental footprint of extraction processes involved in these formulations and to optimize their feasibility at large-scale. There are few published reports of the scale-up of enzyme-assisted (EAE) and microwave-assisted (MAE) extraction methods using grape pomace. Kammerer et al. (2005) designed a pilot-scale enzyme-assisted extraction of polyphenols from 5 kg Cabernet Mitos pomace using 5,000 ppm pectinolytic enzyme and 2,500 ppm cellulolytic enzyme at 50 °C for 2 h. In a related study, Maier et al. (2008) also scaled the enzymatic extraction of Lemberger pomace pigments from 200 g (lab scale) to 5 kg (pilot scale), yielding 91.9% phenolic acids, 92.4% non-anthocyanin flavonoids, and 63.6% anthocyanins using a mixture of pectinolytic and cellulolytic enzymes (ratio 2:1 at 4,500 mg/kg dry matter) at pH 4.0 with constant stirring for 2 h at 40 °C. At commercial scale, however, the processing costs associated with the long extraction times, high enzyme loading, need for additional purification steps, or degradation of the enzyme at extreme conditions may hinder the economic feasibility of this method despite the use of benign solvents like water (Das et al., 2021). A promising technique for addressing the costs of enzyme-assisted extraction, in addition to combining it with other alternative extraction techniques, is enzyme immobilization. This strategy aims to adsorb or entrap the enzyme on a supporting material, like silica or agarose, which allows for the recycling of the enzyme while maintaining or extending its activity even after numerous processing cycles (Karav et al., 2016).

The large-scale feasibility of microwave-assisted extraction (MAE) is less explored in grape pomace feedstocks but has been explored for the extraction of phenolics and/or oils from 4

kg lettuce (Périno et al., 2016), 500 g orange peel (Angoy et al., 2020), and soybean and rice bran (Terigar et al., 2011) using a pilot-scale continuous microwave system. Compared to the conventional hydrodistillation method, the pilot-scale microwave-assisted centrifugation method designed by Angoy et al. (2020) required 25% less energy (16.4 vs. 20.9 MJ/kg fresh material) for the extraction of essential oils from 500 g orange peels. In another study, a life-cycle assessment of microwave-assisted pectin extraction at pilot-scale (20 L) reduced the environmental impact of orange peel processing by 25% compared to traditional hydrochloric acid-assisted thermal processing while also improving pectin yield by 2% (Garcia-Garcia et al., 2019). To prove the industrial feasibility of MAE, it will be crucial to design equipment that can maintain the power of the electromagnetic field with high sample throughput during a continuous process system while simultaneously meeting strict financial budgets.

In addition to the above technical and economic concerns, the transition from benchtop to pilot-scale to commercial-scale production of grape pomace-derived products will also require insight into the delivery mechanisms of the bioactive compounds. For example, to commercialize polyphenolic-enriched foods at large scale, it will be important to consider: the types of food matrix components included in the formulation; the interactions between components in the food; the interactions between food components and grape pomace phenolics; the desired sensory properties of the final food product; and the variety of technologies available to promote stability, bioaccessibility, and bioavailability of the phenolics.

Strategies for enhancing the bioaccessibility (i.e., the ability of phenolics to be released from the food matrix and modified through digestion) of phytonutrients include, but are not limited to, encapsulation, emulsification, roasting, extrusion, nanomilling, enzymatic pretreatment, microfluidization, and co-supplementation (Figure 1.4). Once the phytonutrients

are liberated from the matrix and solubilized within the digestive tract, the bioavailability of the compounds plays a role in ensuring that the compounds will enter the systemic circulation and reach the targeted organ for their intended bioactive role. The bioaccessibility and bioavailability of phenolics can be measured *in vitro* and *in vivo*.

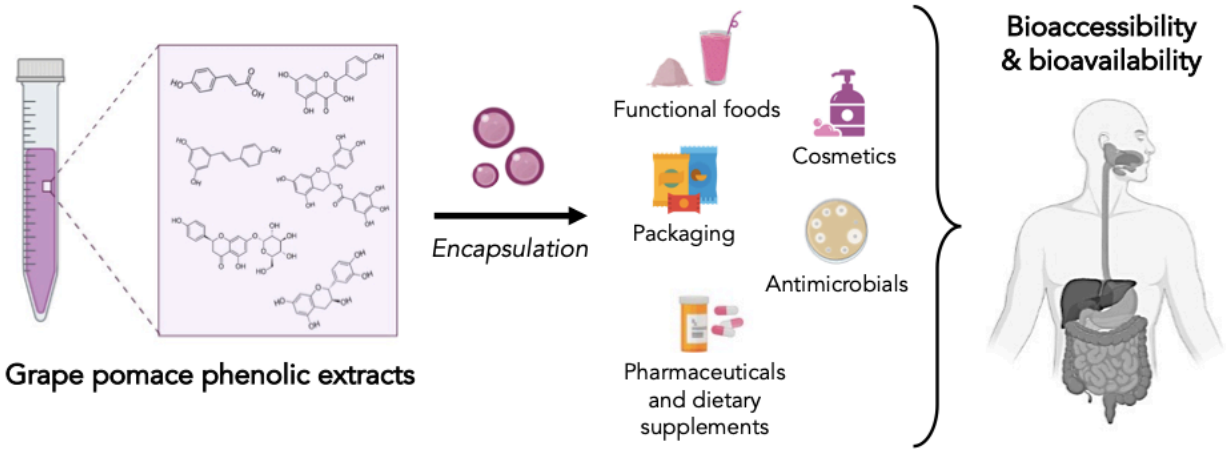

Figure 1.4. Example of downstream processing strategies (e.g., encapsulation) to enhance the bioaccessibility and bioavailability of bioactive ingredients for the development of value-added products using wine grape pomace phenolics. Graphics created with BioRender.com.

Common *in vitro* methods for measuring bioaccessibility include ABTS, ORAC, DPPH, and FRAP assays for measuring antioxidant activity. Through the use of different reagents, chemical mechanisms (e.g., hydrogen atom transfer or electron transfer), and measurement techniques (e.g., absorbance or fluorescence curves), these antioxidant assays create different readouts of radical scavenging activity (Fernández-Fernández et al., 2020). Therefore, multiple antioxidant assays are typically used within one study to provide a more representative overview of the antioxidant capacity of the compounds. Stimulated gastric digestion is another strategy used to measure the permeability of the bioactives throughout an artificial digestive system that mimics the mouth with the use of salivary amylase, the stomach with the use of pepsin at pH 2, the small intestine at a more neutral environment with bile and pancreatin, and eventually the

large intestine where the digested fluid can be used as a substrate for colonic fermentation (Taladrid et al., 2021). To further support the health benefits of plant-derived compounds, *in vivo* animal models are also employed. Animal studies can help provide additional insight into the biological activity of plant secondary metabolites on animal cell viability, solubility, and permeability that can expand upon results from common *in vitro* methods like MTT and Caco-2 assays (Chedea et al., 2018).

To increase the intestinal residence time of grape pomace phenolics and xylooligosaccharides, Costa et al. (2021) successfully designed alginate and chitosan nanoparticles (400–1000 nm) to encapsulate the bioactive compounds for oral delivery systems. In another encapsulation approach, Rubio et al. (2020) used residual Saccharomyces cerevisiae cells to encapsulate Cabernet Sauvignon and Bordeaux grape pomace phenolic extracts. The microcapsules (< 11.45 μm) yielded low water activity (< 0.29), low hygroscopicity (< 13.7 g/100 g), and low moisture content (< 7.10%) to support storage stability while improving the phenolic bioaccessibility by *in vitro* gastrointestinal simulation by 14.3–35.0% compared to the free extracts. The development of novel delivery mechanisms, like encapsulation, is an important step for ensuring the efficacy of grape pomace polyphenolic extracts in biological systems, but determining the exact mechanism of action, the proper dosage, and the price parity of plant-derived bioactives require further exploration.

1.6. Conclusions

Valorizing wine grape pomace has the potential to address critical challenges associated with escalating global supply-chain issues, the agricultural environmental footprint, and the persistence of human health concerns. While the winemaking industry already makes a conscious effort to utilize numerous diversion strategies for its byproducts, there remains an

opportunity for these efforts to: i) reduce the dependence on hazardous, flammable, or toxic materials for processing; ii) utilize the entirety of the waste stream through novel applications following the biorefinery concept; and iii) scale these efforts beyond local viticultural efforts to build widespread awareness of valorization techniques for all agricultural byproducts. This review highlighted the various fractions of winemaking waste streams that can pose functional benefits for food, beverage, packaging, cosmetic, and supplement industries while exploring the current landscape of promising non-conventional extraction techniques to develop feasible strategies capable of enhancing the extraction of grape pomace compounds with the desired composition and biological properties. However, this overarching goal requires a deeper understanding of the impact of these aforementioned strategies on structural composition and biological properties of these bioactive and the optimization strategies available for enhancing process feasibility. In the following Chapters, the development of lab-scale green extraction methods for releasing polyphenols from unfermented Cabernet Sauvignon pomace will be discussed, and the polyphenolic profiles and *in vitro* antioxidant activities will be analyzed to provide a preliminary proof of concept of the biological properties of extracts generated under different processing conditions. While these experiments showcase the positive results from enzyme- and microwave-assisted techniques, future considerations will have to address the pathway for transitioning these strategies away from the bench and towards industrial designs.

1.7. Acknowledgments

BioRender.com graphics were created on March 15, 2022, under license agreement numbers IQ23OH8KMQ (Figure 1.1), AH23OH9XAF (Figure 1.2), and SD23OH926X (Figure 1.4).

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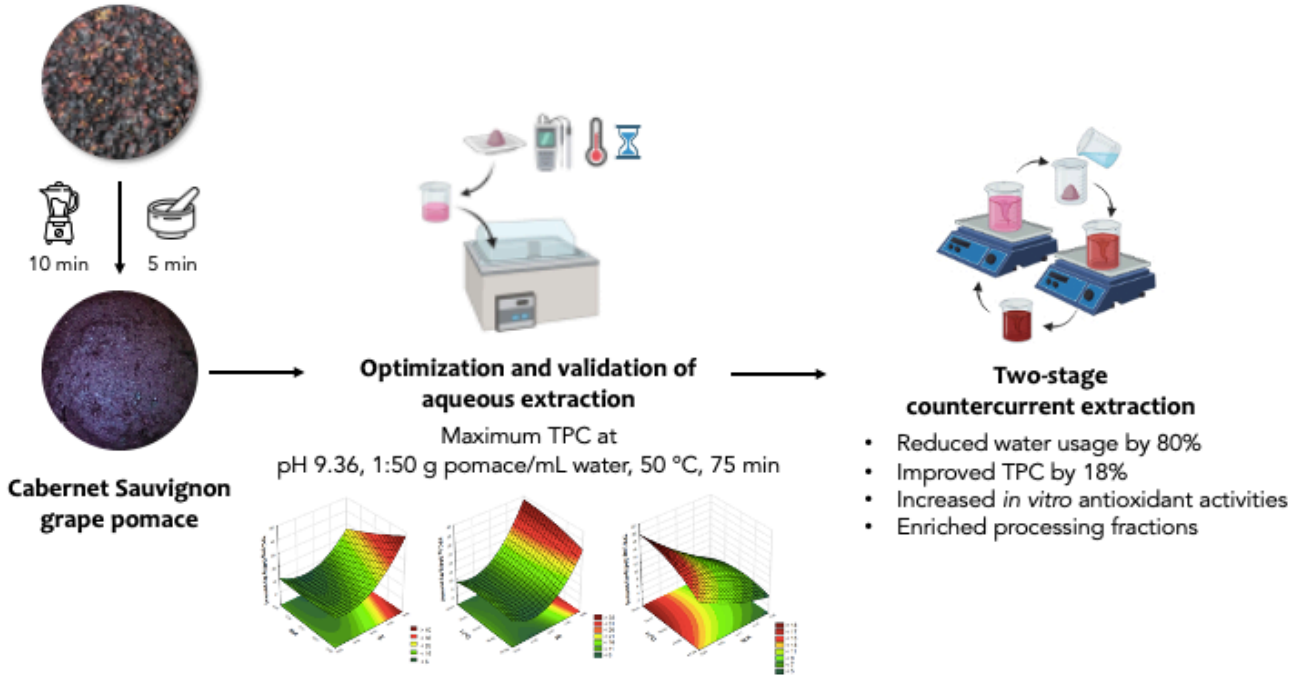
<u>Chapter 2:</u> Revitalizing unfermented Cabernet Sauvignon pomace using an eco-friendly, two-stage countercurrent process: Role of pH on extractability of bioactive phenolics

Graphical abstract



Abstract

Cabernet Sauvignon grape pomace, an underutilized byproduct of the winemaking industry, remains an untapped source of valuable phenolic compounds. The aqueous extraction process (AEP) is an environmentally-friendly strategy that enables the extraction of phenolics from the grape cell-matrix without the use of harsh conventional solvents. To explore the effects of single-stage AEP parameters on the total phenolic content (TPC) of unfermented Cabernet Sauvignon grape pomace extracts, the concurrent impact of pH (2.64–9.36), solids-to-liquid ratio [SLR, g pomace/mL water] (1:50–1:5), and temperature (41.6–58.4 °C) was evaluated by a central composite rotatable design paired with a kinetic study to identify the effect of extraction

time (15–90 min) for each experimental condition. At optimum extraction conditions (pH 9.36, 1:50 SLR, 50 °C, and 75 min), a maximum TPC of 42.9 ± 2.22 mg GAE/g dry pomace was achieved. To reduce water consumption in the single-stage AEP without compromising its extractability, a two-stage countercurrent extraction process was designed using a 1:10 SLR at optimized extraction conditions (pH 9.36, 50 °C, and 75 min). Compared to the single-stage extraction, the countercurrent process increased the TPC of the extracts by 18%, improved the *in vitro* antioxidant activity of the extracts from 332 to 1058 µmol Trolox/g dry pomace (ABTS) and from 547 to 930 µmol Trolox/g dry pomace (ORAC), while reducing water consumption by 80%. Untargeted metabolomics enabled the identification of a diverse pool of phenolics associated with unfermented Cabernet Sauvignon pomace including an increase in the relative contents of flavonol glycosides in the countercurrent extract compared to single-stage AEP extract. This study provides an efficient, eco-friendly processing strategy for improving the extraction of phenolics from unfermented Cabernet Sauvignon pomace while maintaining ingredient bioactivity for potential applications in food, beverage, nutraceutical, and cosmetic industries.

Highlights

- Simultaneous optimization of pH, SLR, temperature, and reaction time enhanced TPC of wine grape pomace extracts
- Alkaline extraction resulted in the greatest recovery of phenolics from the grape cell-matrix
- Countercurrent extraction reduced water consumption by 80% and improved phenolic extraction by 18%
- Antioxidant activities and phenolic profiles were influenced by extraction conditions

Keywords

Grape pomace; aqueous; alkaline; aqueous extraction; countercurrent extraction; phenolics

2.1. Introduction

The state of California is a major global producer of wine grapes and their derived products. In 2020, California crushed over 3.5 million tons of grapes with red wine varieties accounting for over half of the total crush (California Department of Food and Agriculture, 2021). Cabernet Sauvignon grapes accounted for 14.1% of the total grape crush and 27.4% of red wine varieties alone (California Department of Food and Agriculture, 2021). In addition to traditional red and white wines, other styles like rosé and sparkling wines like "blanc de noir" (white wines made from red grapes) diversify wine grape applications (Hidalgo et al., 2004). However, the overall winemaking process generates tremendous sources of agricultural residues: leaves and stems remain after grape harvesting and destemming; seeds, skins, and pulp (collectively termed grape pomace or grape marc) remain after pressing; and lees, a sediment of yeast cells, remains after fermentation (Chowdhary et al., 2021; Oliveira & Duarte, 2016).

It is estimated that the grape pomace fraction represents approximately 20-30% of the total mass of pressed grapes (Saura-Calixto, 2011), which corresponds to 60% of the total winery solid waste (Oliveira & Duarte, 2016). While grape pomace can be diverted for use as animal feed, compost, fertilizer, distilled spirits, or grape seed oil, the associated issues such as poor animal digestibility and low nutrition, environmental toxicity, and processing costs have limited the effectiveness of these applications (Saura-Calixto, 2011). However, even after any of these diversion strategies are employed, a wide variety of valuable components remain in the grape pomace such as dietary fiber, unsaturated fatty acids, proteins, carbohydrates, minerals, organic acids, enzymes, and polyphenols (Chowdhary et al., 2021), evidencing the low biodegradability of grape pomace. Of particular interest for food, beverage, pharmaceutical, nutraceutical, and cosmetic industries are polyphenolic compounds due to their associated antioxidant, anti-

inflammatory, antimicrobial, prebiotic, and anti-carcinogenic properties (Costa et al., 2019; Sinrod et al., 2021; Teixeira et al., 2014). Therefore, the underutilization of wine grape pomace continues to provide opportunities for the development of novel valorization strategies that support economic feasibility and environmental sustainability.

The development of sustainable and effective phenolic extraction methods requires knowledge about the structure, form (i.e., free, esterified, or bound), and location of the compounds in the matrix. Grape pomace phenolics can be chemically bonded to grape cell wall carbohydrates and proteins or physically entrapped within the cellular matrix and other cellular organelles (Pinelo et al., 2006), requiring cleavage of the phenolic-protein or phenolic-polysaccharide complexes formed by hydrogen bonding and hydrophobic interactions. Traditionally, grape pomace bioactive compounds are obtained by multi-step solid-liquid extraction processes using flammable or hazardous solvents like ethanol, methanol, acetone, hexane, ethyl acetate, or combinations of these solvents with water and/or organic acids (Naczk & Shahidi, 2004; Nayak et al., 2018). Acid and alkaline hydrolysis have been used individually and sequentially to target the ester, ether, and glycosidic linkages between polyphenols and cell matrices (Acosta-Estrada et al., 2014; Arranz & Saura Calixto, 2010; Ding et al., 2020). While the use of traditional solvents typically results in substantial polyphenol extraction yields, the long extraction times, high extraction temperatures, and subsequent removal of these solvents for applications in food and drug products can be costly and laborious. With global authorities urgently prompting industries to adopt sustainable practices, integrate upcycling processes, and reduce losses along the supply chain, it is crucial to explore greener extraction methods for utilizing agricultural wastes.

The aqueous extraction process (AEP) is a simple, eco-friendly strategy that can fractionate plant materials into protein-, oil-, and fiber-rich fractions using water as the only solvent (de Moura et al., 2011; Rosenthal et al., 1996). Like conventional solvent extraction, AEP is frequently preceded by mechanical pretreatments such as milling, grinding, and blending to increase the surface area-to-volume ratio and improve the diffusion of compounds into the water (Zhao et al., 2014). Although AEP avoids the use of hazardous solvents, the use of dilute aqueous slurries in single-stage extractions (e.g., 1:50–1:200 g sample/mL solvent) demands additional energy-intensive and expensive concentration steps such as freeze-drying or spray drying to recover the target compounds from the solution. To mitigate the high water consumption needed to achieve high extraction yields in single-stage extractions, multi-stage countercurrent extractions, which involve contacting fresh material with a more saturated solvent from a previous extraction and the almost depleted solids with fresh solvent in a second extraction stage, have been successfully employed to reduce water usage and further increase extractability in soybean and green coffee processing (de Moura & Johnson, 2009; Souza Almeida et al. 2021).

Since aqueous extraction conditions inevitably affect polyphenolic yields and characteristics, the interactive effects of key extraction parameters (e.g., slurry pH, solids-to-liquid ratio, temperature, and time) should be further explored to enhance the recovery of grape pomace phenolics with desired structure-function attributes. Additionally, specific winemaking methods for special products, like white wines made from red grapes, can also impart effects on phenolic profiles and antioxidant activities of the grape pomace extracts by providing a unique composition of tannins, anthocyanins, sugars, proteins, and minerals. Studies evaluating the

aqueous extraction of phenolics from Cabernet Sauvignon pomace collected prior to fermentation have not yet been reported.

Therefore, this study was designed to assess the simultaneous impact of key processing parameters on the extractability of grape pomace phenolics using a single-stage AEP and multi-stage AEP to reduce water usage while minimizing losses. It was hypothesized that optimized extraction conditions of the single-stage AEP could be used as the basis for the development of a multi-stage countercurrent extraction process resulting in a higher concentration gradient to drive molecular diffusion of phenolics to the extraction medium. To accomplish this goal, the present work: i) evaluated the simultaneous effect of pH, solids-to-liquid ratio, temperature, and time on the extractability of total phenolics from unfermented Cabernet Sauvignon grape pomace using single-stage AEP; ii) developed a two-stage countercurrent extraction system to reduce water consumption while maintaining aqueous extraction efficiency; and iii) measured the impact of extraction conditions and processes (single- and multi-stage) on phenolic composition and *in vitro* antioxidant properties of the grape pomace extracts for future food and health applications. An untargeted metabolomics approach was used to characterize the diverse pool of polyphenols associated with unfermented Cabernet Sauvignon pomace.

2.2. Materials and methods

2.2.1. Chemicals and reagents

Folin–Ciocalteu phenol reagent, Trolox®, $K_2O_8S_2$ (potassium persulfate), 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH radical), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS radical), D(+)-glucose, sulfuric acid, phenol, and fluorescein were acquired from Sigma-Aldrich (Saint Louis, MO, USA). Anhydrous sodium carbonate was purchased from VWR Chemicals, BDH® (Solon, OH, USA), and anhydrous gallic

acid was purchased from Chem-Impex International (Wood Dale, IL, USA). The phosphate buffer solution was obtained from bioWORLD (Dublin, OH, USA). Ethanol (95% v/v) was obtained from Decon Labs (King of Prussia, PA, USA).

2.2.2. Raw material

Cabernet Sauvignon grape pomace (*Vitis vinifera* L. cv. Cabernet Sauvignon) was generously provided by the University of California, Davis student winery (Davis, California, USA; approximate latitude: 38.532, longitude: -121.753). Grapes were harvested from the student vineyard in October 2019 and stored at -16 °C until January 2020 when production of a white Cabernet Sauvignon wine began. The grapes were thawed, crushed, destemmed, and pressed (Bucher Xplus 22, Bucher Vaslin North America, Santa Rosa, CA, USA) up to 1.6 psi to avoid excessive color extraction. The juice was subsequently racked and fermented for white wine production and the remaining material—a mix of skins, seeds, pulp, and stems—was stored at -20 °C until July 2020. Then, aliquots of the frozen sample were gathered, and any residual grape stem debris that remained from the destemming process was manually separated from the seeds, skins, and pulp. The seeds, skins, and pulp ("pomace") were homogenized to conform to likely industrial processing conditions. The pomace was blended for 10 minutes in a Vitamix 5200 blender (Vitamix, Cleveland, Ohio, USA) in batches of approximately four cups at a time then stored in bulk at -20 °C until further use. Prior to extraction, aliquots of the bulk frozen pomace were defrosted for 20 minutes at room temperature then ground to a paste using a mortar and pestle for approximately 5 minutes until fully homogenized. The grape pomace material was used fresh, without the use of a drying step as a pre-treatment, to reduce overall energy consumption and prevent potential degradation of phenolics through additional heating.

Proximate composition of the grape pomace was determined using standard methods. Total solids content was determined using AOAC Method 925.09 (AOAC, 2015), total ash by AOCS Method Ba 5a-49 (AOCS, 2017), and total lipids by AOAC Method 922.06 (AOAC, 2012). Total protein content was determined using the Dumas combustion method (Vario MAX cube, Elementar Analysensysteme GmbH, Langenselbold, Germany) with a nitrogen conversion factor of 6.25. Overall, the grape pomace contained 57.80 ± 0.09% moisture, 2.86 ± 0.07% protein, 1.87 ± 0.02% ash, 1.03 ± 0.09% lipids, and 36.4% carbohydrates (measured by difference).

2.2.3. Initial evaluation of the effects of the extraction pH on the aqueous extraction process (AEP) of grape pomace

To aid in the selection of extraction parameters studied in the AEP optimization experiments, the effect of slurry pH on total phenolic content was initially evaluated in a univariate study with pH values varying from pH 2–8. Grape pomace samples were dispersed in deionized water and the slurry pH was adjusted to 2, 4, 6, and 8 with dropwise (< 2 mL) additions of 0.5 M HCl or 0.5–1.0 M NaOH. The solids-to-liquid ratio (SLR) was maintained at 1:10 g pomace/mL water. Triplicate extractions were performed at each pH condition in a 50 °C water bath for 90 minutes with constant stirring at 185 rpm on a magnetic stir plate (Cimarec™ i Telesystem Multipoint Stirrers, Thermo Scientific, Waltham, MA, USA). After extraction, the solids were separated by centrifugation at 4000 rpm for 10 min at 22 °C followed by filtration (Whatman Grade 1 filter papers, Sigma-Aldrich, Saint Louis, MO, USA). Extracts were stored at -20 °C until further analysis.

2.2.4. Effect of conventional solvent extraction on TPC

Solid-liquid extractions were performed to determine the effect of conventional hydroethanolic solvent extraction on the total phenolic content of the grape pomace extracts.

Several solvent ratios were prepared by mixing 95% (v/v) ethanol in deionized water at 0, 20, 40, 60, 80, and 100% ethanol concentrations (v/v). The pH of pure deionized water and 95% ethanol were 4.87 and 5.45, respectively, while the pH of the hydroethanolic slurries (20–80% ethanol, v/v) were 3.85, 4.28, 4.77, and 5.68, respectively. Extractions and separations were performed in triplicate as described for the AEP (Section 2.3) (1:10 SLR at 50 °C with 175 rpm agitation for 90 min), and the extracts were stored at -20 °C until further analysis. To better compare the effectiveness of the conventional solvent extraction with the AEP, triplicate hydroethanolic extractions were also performed as described above at the optimum extraction conditions identified in the single-stage AEP (1:10 SLR, 50 °C, 75 min) using the selected 60% ethanol.

2.2.5. Understanding the simultaneous effect of extraction parameters in the single-stage AEP of grape pomace

The results of the preliminary pH screening in the AEP (Section 2.3) guided the selection of the broader pH range explored in the AEP optimization design. The concurrent effects of pH (2.64, 4.00, 6.00, 8.00, and 9.36), SLR (1:50.0, 1:17.7, 1:9.1, 1:6.1, and 1:5.0), and temperature (41.6, 45.0, 50.0, 55.0, and 58.4 °C) (Supplementary Materials—Table S2.1) on total phenolic content of single-stage AEP grape pomace extracts were evaluated by a central composite rotatable design totaling 17 experimental conditions (2^3, with three repetitions in the central points and six axial points).

A kinetic study was performed for each extraction condition to determine the role of reaction time on phenolic extractability. During extraction, aliquots were collected at 15, 30, 45, 60, 75, and 90 min and immediately placed in an ice bath to inhibit further extraction. The total phenolic content was measured at each time point to develop corresponding reaction curves.

Extractions were performed by dispersing the grape pomace sample in 10 mL nanopure water to achieve the SLR determined by the experimental design (Supplementary Materials—

Table S2.1) (Figure 2.1). Slurry pH and reaction temperature were also adjusted according to the experimental design. After extraction, the slurry was centrifuged to separate the grape pomace extract as described in the above sections.

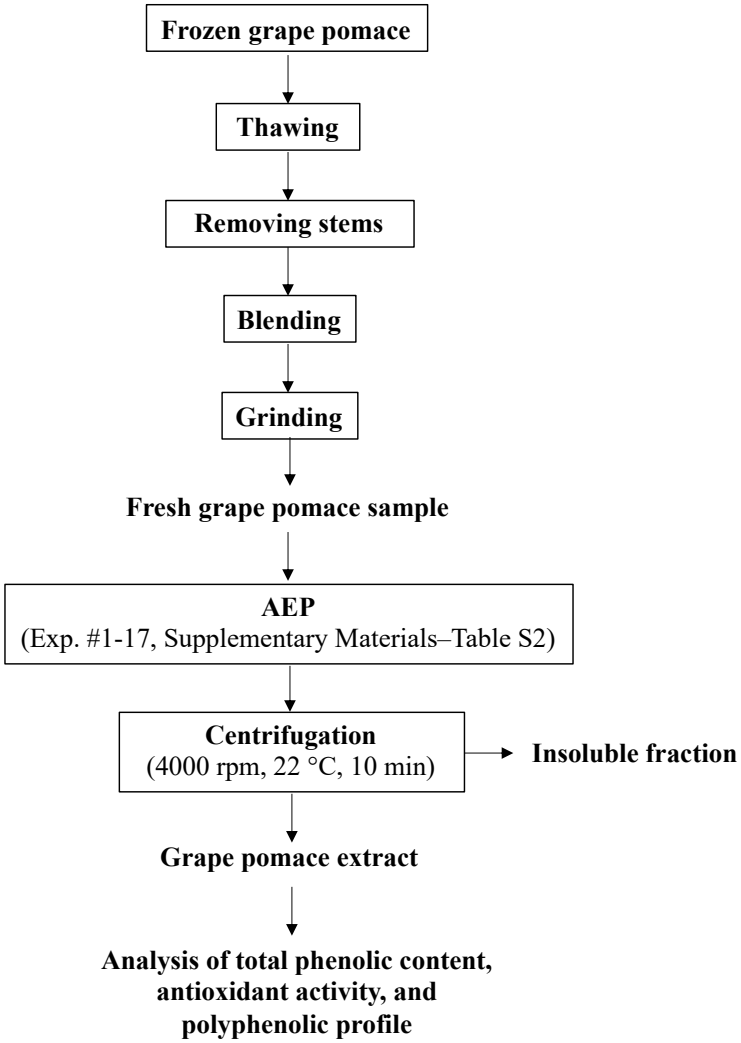


Figure 2.1. Process flow diagram for the single-stage AEP experiments evaluating the effect of pH (2.64–9.36), SLR (1:50.0–1:5.0), and temperature (41.6–58.4 °C) on grape pomace phenolic extraction.

2.2.6. Two-stage countercurrent AEP of grape pomace

To circumvent one of the main challenges of aqueous extraction processes, which is the use of large volumes of water to drive extraction, a two-stage countercurrent extraction process

was developed for grape pomace to achieve high extraction yields with reduced water consumption (Figure 2.1). In this approach, a saturated phenolic extract resulting from a previous extraction was mixed with the addition of fresh grape pomace, while in the second extraction, fresh water was used to slurry the nearly depleted solids (insoluble fraction) produced from the first extraction. Based on the optimum extraction conditions identified for phenolic extraction using the single-stage AEP (pH 9.36, 1:50 SLR, 50 °C, 75 min) and the expected improvement in extractability when using higher SLR in the countercurrent approach, an intermediate SLR (1:10) and a shorter extraction time (45 min) were selected for the first extraction of the countercurrent process.

Each two-stage AEP trial was composed of three two-stage sequential extractions to enable adequate recycling of the extracts coming from the previous extraction. The entire countercurrent process was completed in a single day to prevent freeze-thaw cycles of the grape pomace material. Briefly, 1 g of freshly ground grape pomace was dispersed in water to achieve a 1:10 SLR, and the slurry pH was adjusted to 9.36. The first extraction was performed at 50 °C under constant stirring at 300 rpm for 45 min. The slurry was then centrifuged at 4000 rpm for 10 min at 22 °C to separate the solids (insoluble fraction A) from the extract (Figure 2.2). Subsequently, the insoluble fraction A was then dispersed into fresh water to achieve a 1:10 SLR in the second extraction (pH 9.36, 50 °C, 75 min). After extraction, the slurry was centrifuged to obtain extract B, which was then recycled as the solvent for the next extraction with fresh grape pomace. This two-step extraction was repeated two more times to complete one two-stage countercurrent trial. AEP trials were replicated three times and only samples collected from the last extraction of each AEP trial were analyzed (extract A).

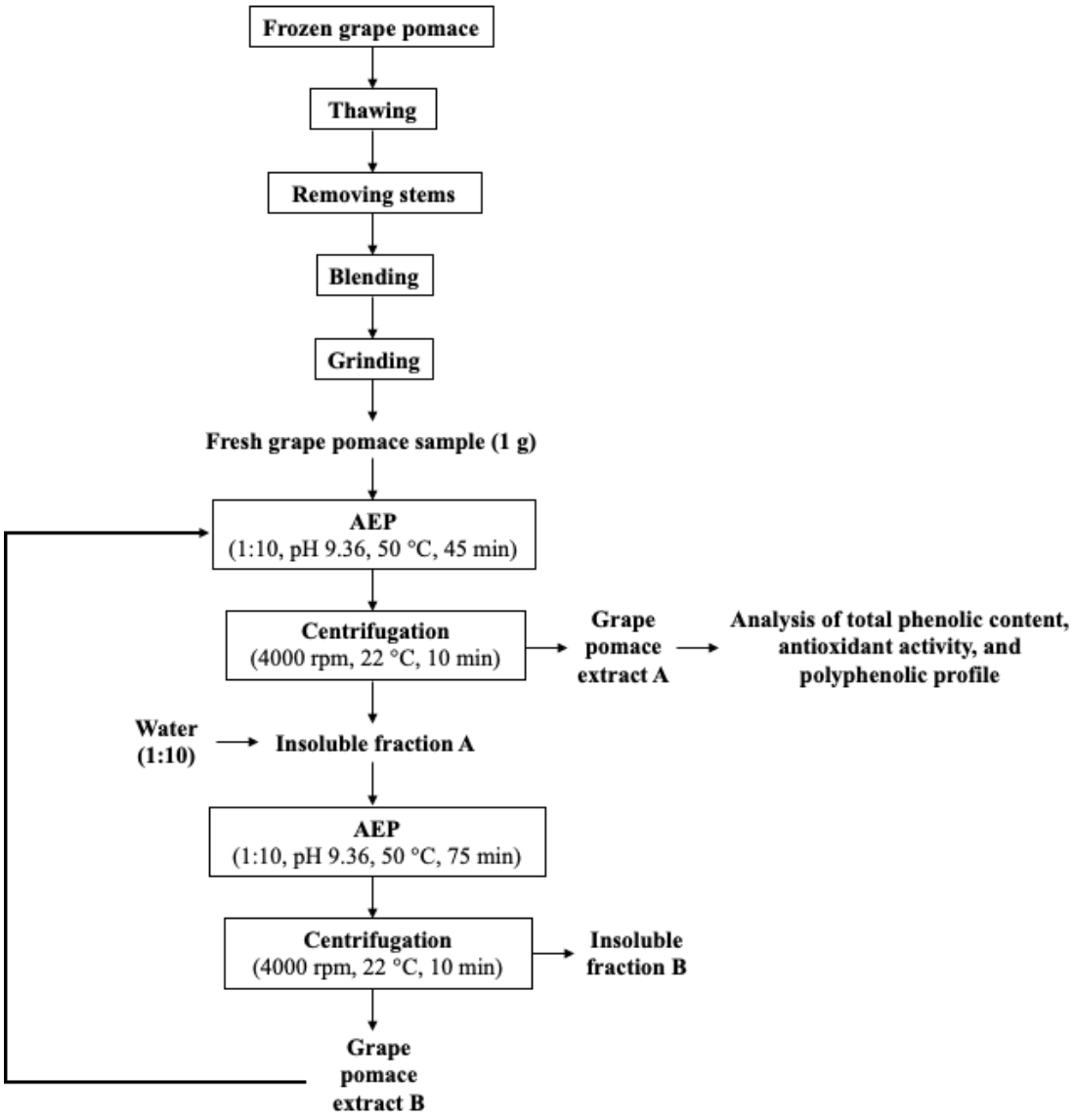


Figure 2.2. Process flow diagram for the two-stage countercurrent AEP

2.2.7. Chemical analysis of extracts

2.2.7.1. Total phenolic content (TPC)

The total phenolic content of the extracts was determined according to the Folin-Ciocalteu spectrophotometric method as described by Singleton et al. (1999). An aliquot of 25 µL of the grape pomace extracts (diluted 1:10, v/v, or 1:25, v/v, in nanopure water) and 125 µL of Folin-Ciocalteu:water solution (1:10, v/v, in nanopure water) were transferred to a clear 96-

well microplate. The mixture was agitated at 300 rpm for 5 minutes at 37 °C. Next, 100 µL of 7.5% (w/v) anhydrous sodium carbonate solution was added and the mixture was agitated (300 rpm, 30 min, 37 °C) then kept at rest for 90 min in the dark at 37 °C. The absorbance was read at 760 nm using a microplate reader (SpectraMax iD5, Molecular Devices, San Jose, CA) and results were calculated using a standard curve of anhydrous gallic acid ($R^2 = 0.999$) with known concentrations of 5–95 µg/mL. Results were expressed as mg of gallic acid equivalent (GAE)/g dry grape pomace extract. For the preliminary tests, duplicate measurements of each triplicate extract were performed (n = 6). For the optimization and kinetics experiments, triplicate measurements of each triplicate sample were performed (n = 9).

2.2.7.2. ABTS and ORAC assays for antioxidant capacity of the extracts

The ABTS assay for radical cation scavenging activity was performed as described by Al-Duais et al. (2009) with some modification. The radical stock solution was produced by mixing 38.4 mg ABTS and 6.62 mg $K_2O_8S_2$ (potassium persulfate) in 10 mL deionized water. The radical solution was incubated overnight at room temperature in the dark. After incubation, the radical stock solution was diluted with 95% (v/v) ethanol to obtain an initial absorbance of 0.70 ± 0.20 at 730 nm. The analysis was performed by pipetting 20 µL of each grape extract sample (diluted 1:30, v/v, in ethanol) into a clear 96-well microplate followed by the addition of 200 µL of fresh diluted $ABTS^{\bullet+}$ solution. A 20 µL sample of ethanol with 200 µL $ABTS^{\bullet+}$ solution was used as the control. The mixtures were agitated at 300 rpm for 6 min, then the microplate was read at 730 nm using a spectrophotometer (SpectraMax iD5; Molecular Devices, San Jose, CA, USA). A calibration curve ($R^2 = 0.98$) using Trolox standard solutions (80–340 µM, diluted in ethanol) was used to calculate the results as µmol Trolox equivalent (TE)/g dry pomace.

The ORAC assay was performed according to the method described by Zulueta et al. (2009). Briefly, 50 µL of the control (phosphate buffer solution, pH 7.0, 75 mM), grape pomace extract (diluted 1:2000, v/v, in PBS), or standard (Trolox, 20 µM, diluted in PBS) were added to the wells of a black 96-well microplate. A sample of 50 µL of fluorescein (78 nM, diluted in PBS) was added, and the plate was agitated at 300 rpm for 15 min at 37 °C followed by the addition of 25 µL of 221 mM AAPH radical solution. The plate was read using a microplate reader (SpectraMax iD5; Molecular Devices, San Jose, CA, USA) warmed to 37 °C, and set at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The fluorescence measurements were read every 5 min for 1 h. The results were calculated using Equation 1.1 and were converted to µmol Trolox equivalent (TE)/g dried pomace.

$$\mu\text{M Trolox} = \frac{C_{\text{Trolox}}(AUC_{\text{sample}} - AUC_{\text{blank}}) * DF}{AUC_{\text{Trolox}} - AUC_{\text{blank}}} \quad \text{(Equation 1.1)}$$

Where C_{Trolox} represents the concentration of the standard (20 µM), DF is the sample dilution factor (2000) and AUC represents the areas below the fluorescence decay curve of the sample, blank, or Trolox standard.

2.2.7.3. Untargeted phenolic profiling of the grape pomace extracts

Identification of grape pomace extract phenolic compounds was performed by the West Coast Metabolomics Center Central Services Core (UC Davis, Davis, CA, https://metabolomics.ucdavis.edu/core-services/assays-and-services) using a Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Scientific, Waltham, MA, USA). Polyphenol identification and data processing were performed as described by Fiehn et al. (2008). A series of internal standards were added to the samples: Caffeine-d9, CUDA, daidzein-d4, genistein-d4, trans-cinnamic acid-d5, and hippuric acid-d5. A concentration of 0.5 µg/mL of hippuric acid-d5 was used with all other standards concentrated at 1 µg/mL in resuspension mix.

Samples were resuspended to 100 µL with 0.5 µL injected for ESI positive mode and 5 µL for ESI negative mode (Supplementary Materials—Table S2.6). The reported peak heights were determined by dividing each metabolite peak by the sum of all peak heights for all identified metabolites for each sample.

2.2.8. Statistical analysis

All extractions and biological assays were performed in at least triplicate with the results expressed as mean ± standard deviation (SD) of the replicates. Analysis of variance (ANOVA) followed by Tukey HSD test was performed to determine significant differences among experiments at $p < 0.05$ using the Astatsa (2016, Navendu Vasavada) online program. For the CCRD optimization experiments, the R^2 and F-value for regression model significance were generated using the Protimiza Experimental Design® Software (http://experimentaldesign.protimiza.com.br). Statistica® (Version 13.3, TIBCO Software Inc., Palo Alto, CA, USA) was used to generate the response surface plot (Figure 2.5) and to determine ANOVA significance testing at $p < 0.05$. JMP® (Trial 16.1.0, serial number T-TYPQDH0JJC, SAS Institute Inc., Cary, NC, USA) was also used for ANOVA significance testing at $p < 0.05$.

2.3. Results and discussion

2.3.1. Effect of extraction pH on TPC of AEP grape pomace extracts

The effect of aqueous solvent pH on phenolic extractability has been minimally explored with varying results depending on the type of solvent used. The effect of slurry pH in the AEP was evaluated using a 1:10 SLR at 50 °C for 90 min. Overall, TPC of the extracts significantly increased as the extraction pH increased from 2 to 8 (Figure 2.3A). The maximum TPC of 11.77 ± 0.33 mg GAE/g dry pomace was achieved at pH 8 (Figure 2.3A) with no significant difference

($p > 0.05$) between the TPC of extracts obtained at pH 2 (7.33 ± 0.49 mg GAE/g dry pomace) and pH 4 (6.77 ± 0.59 mg GAE/g dry pomace). However, both acidic extraction conditions resulted in extracts with significantly lower TPC than the ones obtained at pH 6 (8.57 ± 0.09 mg GAE/g dry pomace) and pH 8 (11.77 ± 0.33 mg GAE/g dry pomace). These results suggest that aqueous extraction at alkaline pH has a significant role in improving the phenolic extractability from grape pomace.

Our results are in agreement with the literature, where Librán et al. (2013) evaluated the effects of a range of extraction pH (2, 5.33, 8.66, and 12) and hydroethanolic concentrations (0, 25, 50, 75, and 100% ethanol, v/v) on the extraction of polyphenols from Tempranillo grape pomace at 25 °C and incubation time of 2 h. At 0% ethanol, a five-fold increase in TPC was observed when the pH of the extracting media (i.e., water) was adjusted from pH 2 (4.58 mg GAE/g dry sample) to pH 12 (28.06 mg GAE/g dry sample). However, at higher ethanol concentrations (50, 75, and 100%), alkaline conditions generated significantly ($p < 0.01$) lower TPC compared with acidic conditions. It is possible that the use of alkaline pH at high hydroethanolic concentrations may degrade phenolic acids due to the low solubility of the carboxylic acid groups in their anionic forms (Oreopoulou et al., 2019).

The phenolic extraction mechanism under alkaline conditions is believed to target the release of bound phenolics from the plant cell wall by cleaving ester and ether linkages to polysaccharides, fibers, lignin, and proteins (Arranz et al., 2009). On the other hand, acid hydrolysis targets glycosidic linkages to phenolics but typically leaves ester linkages intact (Acosta-Estrada et al., 2014). Because of the key role of pH on enhancing TPC in the AEP, the simultaneous effects of a wider pH range, SLR, and temperature was further evaluated for the single-stage AEP.

2.3.2. Effect of ethanol concentration on TPC of conventional solvent extracts

Hydroethanolic solvents (0, 20, 40, 60, 80, and 100% ethanol in water) were evaluated in parallel for their effect on the TPC of grape pomace to better understand the yields and trends of conventional solvent extraction compared to AEP. The use of mono-component solvents produced the lowest phenolic yields (Figure 2.3B), while increasing ethanol concentrations from 0 to 60% resulted in increasing TPC of the grape pomace extracts (8.31 ± 0.11 to 27.48 ± 0.96 mg GAE/g dry pomace) with no significant difference in TPC between 40 and 60% ethanol (Figure 2.3B). It is likely that increasing ethanol concentrations up to 60% promoted phenolic extraction by enhancing solvent permeability and solubility of the grape skin and seed phenolics with the addition of heat (50 °C). Compared to the AEP at pH 8, which resulted in an extract TPC of 11.77 ± 0.33 mg GAE/g dry pomace, the use of 60% ethanol (v/v) at acidic pH conditions increased the TPC of the extract to 27.48 ± 0.96 mg GAE/g dry pomace. However, at ethanolic concentrations beyond 60%, the TPC of the extracts declined with the decrease in solvent polarity to 12.98 ± 0.32 mg GAE/g dry pomace at 100% ethanol (v/v).

A hydroethanolic mixture of 60% ethanol (v/v) was therefore selected as the representative conventional solvent for this study, which reflects findings in the literature that report improvements in phenolic extraction at hydroethanolic mixtures at or above 50% ethanol. In a previous study, a maximum TPC of 28 mg GAE/g dried Muscadine seed powder was achieved when using 50, 60, or 70% ethanol (1:10 SLR, 30 min, ambient temperature) (Yilmaz & Toledo, 2006).

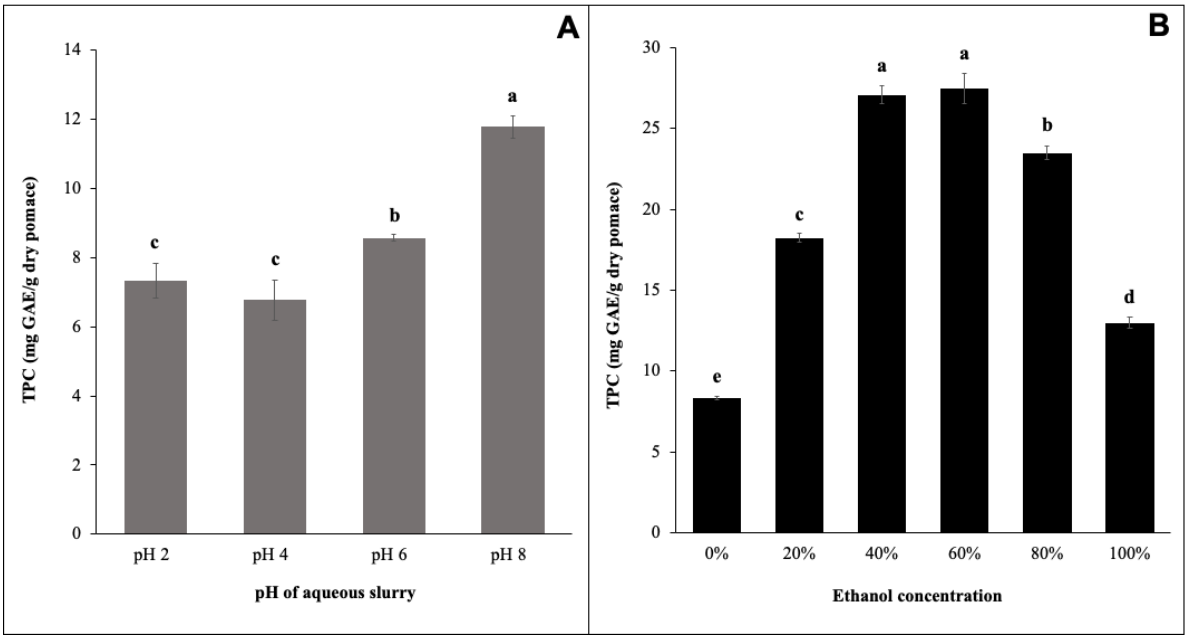


Figure 2.3. Effects of extraction pH on the TPC of AEP extracts (A) and ethanol concentration on the TPC of extracts produced by conventional solvent extraction (B). Extractions were conducted using a 1:10 SLR at 50 °C for 90 min. Different letters represent significant differences in TPC ($p < 0.05$) across the extraction treatments. Data represent the mean ± one standard deviation of triplicates.

2.3.3. Simultaneous effect of extraction parameters on TPC of single-stage AEP

The results of the preliminary aqueous extraction exploring the effect of slurry pH helped guide the selection of the pH range for the CCRD optimization study, while the SLR and temperature parameters were determined by a review of the literature. The CCRD evaluated the effects of extraction pH, solids-to-liquid ratio, and temperature while the kinetic study evaluated the role of the extraction time on phenolic extractability (Figure 2.4). Overall, the TPC of AEP extracts ranged from 5.10 ± 0.03 (Experiment #12 at 15 min) to 36.76 ± 0.55 mg GAE/g dry pomace (Experiment #10 at 90 min) (Figure 2.4).

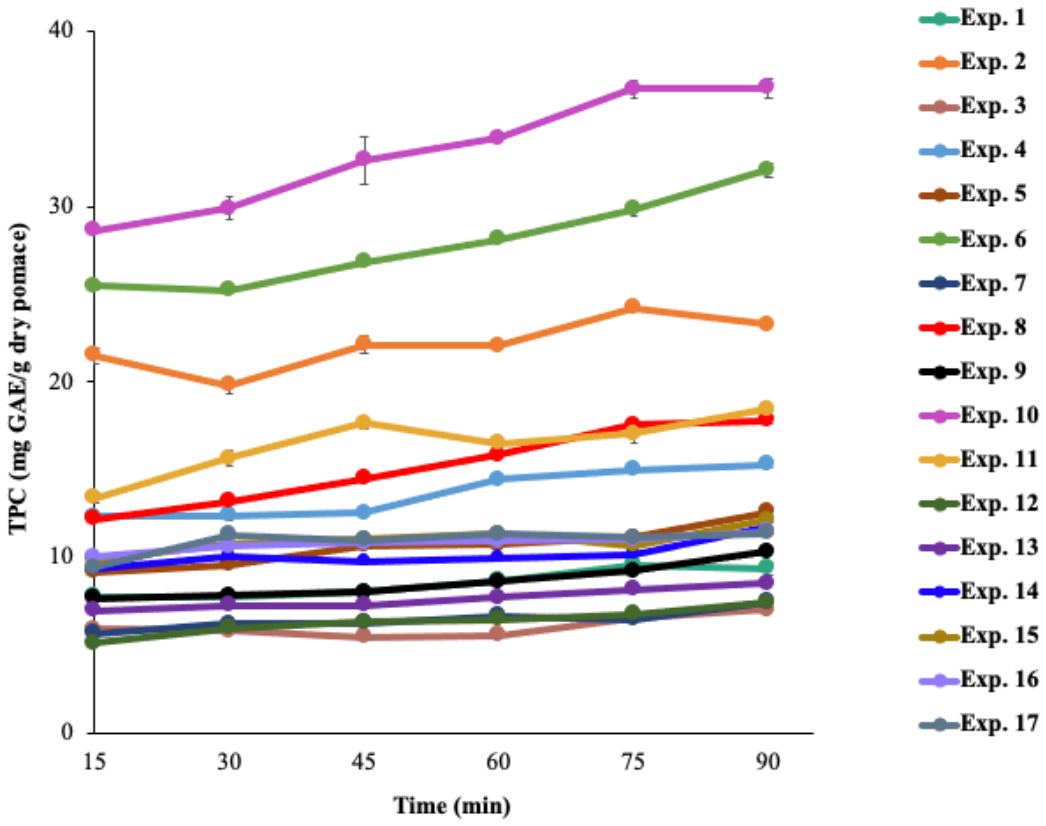


Figure 2.4. Simultaneous impact of pH (2.64–9.36), SLR (1:5.0–1:50.0), and temperature (41.6 °C–58.4 °C) on the TPC of grape pomace extracts from the single-stage AEP at different reaction times. Data represent mean ± SD of triplicate extractions.

Regardless of the extraction time from 15 to 90 min, three experimental conditions yielded high total phenolic contents: Experiment #10 (28.64–36.76 mg GAE/g dry pomace, pH 9.36, 1:9.1 SLR, 50 °C), Experiment #6 (25.47–32.09 mg GAE/g dry pomace, pH 8.00, 1:17.7 SLR, 55 °C), and Experiment #2 (21.51–23.27 mg GAE/g dry pomace, pH 8.00, 1:17.7 SLR, 45 °C) (Supplementary Materials—Table S2.2). Similarities between these experiments highlight the role of alkaline conditions in phenolic extraction, and as a result, may suggest that the use of alkaline pH can compensate for changes in the other processing conditions like lower temperatures. In another study evaluating grape marc phenolic extraction kinetics using 50%

ethanol, the equilibrium concentration was achieved after 60 min of extraction at 40 °C, 45 min at 50 °C, and 30 min at 60 °C using a 1:50 SLR (Sant'Anna et al., 2012). These results suggest that lower temperatures required longer extraction times to reach equilibrium, yet the results of the present study suggest that the pH parameter may play a stronger role than extraction time and temperature in increasing phenolic yields. Analysis of the extraction kinetics in Figure 2.4 and the TPC in Supplementary Materials—Table S2.2 shows a general upward trend in the TPC of grape pomace extracts up to 75 min with a limited change in phenolic extractability at 90 min. Therefore, it was concluded that the magnitude of any TPC increase from 75 min to 90 min was too small to warrant the additional 15 min of extraction time. Based on these results, 75 min was selected as the extraction time at which to develop the regression model and response surface plot.

The regression model at 75 min [TPC (mg GAE/g dry pomace) = $10.72 + 7.26x_1 + 4.33x_1^2 - 3.41x_2 + 0.95x_3 - 1.73x_1x_2$] was reparametrized to include only variables significant at $p < 0.05$ (Figure 2.5). The regression model [$F_{calculated}$ (94.3) > $F_{tabulated}$ (3.2)] and F-test [$F_{calculated}$ (53.1) > $F_{tabulated}$ (19.4)] were both statistically significant at $p < 0.05$ (Supplementary Materials—Table S2.3). The lack of fit in the model can be explained by the small variation in the replicates (central points), which causes a very low pure error. The regression model was able to explain 98% of the variation between the predicted and experimental TPC at 75 min, and experimental yields were relatively similar to the predicted yields. The TPC from all CCRD experiments at 75 min was compared with the predicted values using the regression model (Supplementary Materials—Table S2.4). At extraction conditions resulting in higher phenolic yields, a low percent error (below |9%|) was observed, which supports the high predictability of the model within the range evaluated.

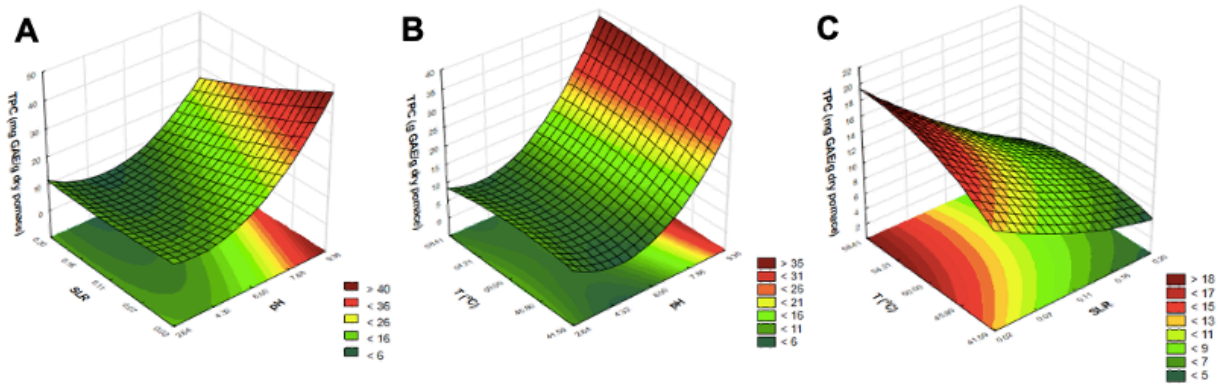


Figure 2.5. Response surface plot showing the effect of (A) SLR vs. pH, (B) Temperature (°C) vs. pH, and (C) Temperature (°C) vs. SLR on the TPC of grape pomace extracts in single-stage AEP after 75 min

The total phenolic content of the grape pomace extracts was largely influenced by pH, specifically at more alkaline pH, as observed by its large and positive coefficient (x_1). The substantial impact of alkaline pH on TPC is also reflected by the quadratic term (x_1^2). Previous literature exploring the use of alkaline hydrolysis for the extraction of grape pomace phenolics is scarce, although there are reports of using alkaline conditions paired with solvent purification to improve phenolic extraction from other agricultural byproducts like apple pomace and cranberry pomace (Li et al., 2020; White et al., 2010). Alkaline conditions have been shown to release bound phenolics by cleaving linkages between plant cell wall carbohydrates and structural proteins, which creates a more porous cell structure that aids in the solubilization of phenolics into the surrounding solvent (Shahidi & Yeo, 2016). Despite the benefits of using alkaline conditions to release bound phenolics, the time of extraction reported in previous studies is lengthy, which hinders the feasibility of this technique at the industrial scale. For this reason, the selected AEP extraction time of 75 min is advantageous compared to previous literature that recommends exposure of grape pomace to 0.01 M NaOH for 24 h at room temperature (Campos et al., 2021). In addition, although some polyphenols have shown degradation at elevated pH

conditions due to oxidation (Friedman & Jürgens, 2000), it is apparent that the benefits of alkaline hydrolysis at optimal conditions can compensate for potential losses in phenolic yields.

Since grape pomace polyphenols are heat-sensitive compounds that are vulnerable to oxidative degradation during extended extraction periods, the effect of extraction temperature was also explored. The role of extraction temperature seemed to be outweighed by the influence of pH and solids-to-liquid ratio based on the results from the regression analysis of the data. Its small coefficient (x_3) suggests a weaker influence of extraction temperature on TPC within the range evaluated. Higher temperatures are responsible for enhancing mass transfer, cell wall permeability, and solubility of target compounds, thus reducing solvent viscosity and in turn shortening extraction times (Oreopoulou et al., 2019). However, the extraction yields of certain polyphenols and their biological properties can begin to decline at mild temperatures around 60 °C (Liyanapathirana & Shahidi, 2005). Even extraction pre-treatments like drying have reduced total extractable polyphenols in grape pomace at temperatures of 100 and 140 °C (Larrauri et al., 1997). On the other hand, temperatures that are too low can hinder mass transfer, require lengthy extraction timelines, and increase the decomposition of polyphenols by oxidation (Spigno et al., 2007). Therefore, the selection of 50 °C is consistent with previous studies that support moderate heat applications for phenolic extraction.

Finally, our results support the use of low SLRs (reduced solids, more water), as evidenced by the moderately large and negative coefficient of X_2 (SLR), to improve phenolic extractability. The beneficial effect of reduced SLR on phenolic extraction has been attributed to the accelerated mass transfer caused by the large concentration gradient between solvent and solute (Oreopoulou et al., 2019). The results of the present study show that the grape pomace slurries that were more concentrated (i.e., 1:5 g pomace/mL water) produced extracts with lower

phenolic content in part due to a lower concentration gradient and slower diffusion of solutes to the solvent. The selection of the 1:50 SLR as the optimal condition agrees with previous literature (Nayak et al., 2018). Importantly, the interaction of both pH and SLR was significant at $p < 0.05$, which reinforces the positive influence of both dilute solutions and alkaline pH on TPC. The response surface plot (Figure 2.5) illustrates that a wide temperature range (e.g, 40–60 °C) is favorable for improving phenolic yields as long as the pH of the solution is strongly alkaline (pH > 9.0) and the SLR is small (SLR < 0.03, diluted solution). According to the regression model, the best conditions were identified as pH 9.36, 1:50 SLR, 50 °C, and 75 min (Figure 2.5). To validate the adequacy of the predictive model, the optimal single-stage AEP condition (pH 9.36, 1:50, 50 °C, 75 min) was validated in triplicate. Experimental validation yielded a TPC of 42.9 ± 2.22 mg GAE/g dry pomace, which was similar to the TPC predicted by the regression model (45.8 mg GAE/g dry pomace).

In comparison to the maximum TPC from the CCRD (36.76 ± 0.55 mg GAE/g dry pomace at pH 9.36, 1:9.1 SLR, 50 °C, and 90 min), the optimized extraction condition improved yields and reduced extraction time by approximately 17%. However, under optimum conditions, a more dilute solution was required to achieve high TPC with respect to the initial condition, requiring over a five-fold increase in the volume of water used. For this reason, a two-stage countercurrent extraction process was performed to determine the efficacy of reducing water consumption without diminishing the phenolic yields.

2.3.4. Selecting the SLR for the development of the multistage countercurrent AEP

Because increasing SLR led to reduced phenolic extraction in the single-stage AEP, the predictive model generated for the single-stage AEP was used to determine a theoretical TPC of extracts at additional SLRs within the range evaluated in the experimental design. Extraction pH

(9.36, coded variable = 1.68) and temperature (50 °C, coded variable = 0) were kept fixed, while the SLR varied.

Table 2.1. Effect of increasing the slurry SLR on the predicted TPC of aqueous extracts produced by the single-stage AEP

	Validated single-stage AEP conditions	Theoretical single-stage AEP at varying SLR conditions								
SLR (1:x)	**1:50**	**1:45**	**1:40**	**1:35**	**1:30**	**1:25**	**1:20**	**1:15**	**1:10**	**1:5**
Uncoded SLR	0.020	0.022	0.025	0.029	0.033	0.040	0.050	0.070	0.100	0.200
Coded SLR	-1.68	-1.64	-1.59	-1.52	-1.43	-1.31	-1.12	-0.802	-0.187	1.68
10.72	10.7	10.7	10.7	10.7	10.7	10.7	10.7	10.7	10.7	10.7
$7.26x_1$	12.2	12.2	12.2	12.2	12.2	12.2	12.2	12.2	12.2	12.2
$4.33x_1^2$	12.3	12.3	12.3	12.3	12.3	12.3	12.3	12.3	12.3	12.3
$-3.41x_2$	5.7	5.6	5.4	5.2	4.9	4.5	3.8	2.7	0.6	-5.7
$0.95x_3$	0	0	0	0	0	0	0	0	0	0
$-1.73x_1x_2$	4.9	4.8	4.6	4.4	4.2	3.8	3.3	2.3	0.5	-4.9
Predicted TPC	45.8	45.6	45.2	44.8	44.2	43.5	42.3	40.3	36.4	24.6

As observed in Table 2.1, the theoretical phenolic extraction decreases from 45.8 to 24.6 mg GAE/g dry pomace when the SLR increases from 1:50 to 1:5. Based on the predicted TPC of the nine theoretical scenarios, a SLR of 1:10 was selected for the two-stage countercurrent extraction process. The predicted TPC of a single-stage AEP using a 1:10 SLR was 36.4 mg GAE/g dry pomace, which offers an 80% reduction in water compared to the 1:50 SLR used in the optimized extraction condition while still maintaining impressive phenolic yields. It was predicted that the use of the lowest SLR, 1:5 g pomace/mL water, would generate a highly viscous slurry that could impede the diffusion of compounds into the solvent due to solvent saturation. Therefore, a 1:10 SLR was used in the development of the two-stage countercurrent extraction to determine whether this reduction in water usage would impact phenolic yields.

2.3.5. Two-stage countercurrent extraction of phenolics from grape pomace

For the two-stage countercurrent AEP, extractions were performed at pH 9.36, 1:10 SLR, 50 °C, and 45 min in the first extraction followed by 75 min in the second extraction. The total

phenolic content of the extracts from the two-stage countercurrent AEP are shown in Table 2.2 in comparison to the single-stage AEP and a conventional solvent control. The TPC of the two-stage countercurrent AEP (50.5 ± 1.16 mg GAE/g dry pomace) was significantly greater ($p < 0.05$) than the yield from the optimized single-stage AEP (42.9 ± 2.22 mg GAE/g dry pomace) but significantly lower than the TPC of the extract from conventional solvent extraction (62.5 ± 1.27 mg GAE/g dry pomace). Overall, the two-stage countercurrent AEP resulted in an 18% increase in TPC of the extracts, compared to the single-stage AEP, while simultaneously reducing water consumption by 80%.

Table 2.2. TPC and antioxidant activities of validated single-stage AEP, two-stage countercurrent AEP, and conventional solvent extraction

	TPC (mg GAE/g dry pomace)		ABTS (µmol TE/g dry pomace)	ORAC (µmol TE/g dry pomace)
	Predicted*	Experimental**		
Validated single-stage AEP pH 9.36, 1:50 SLR, 50 °C, 75 min	45.8^{a}	42.9 ± 2.22^{bC}	332 ± 10.1^{C}	547 ± 44.5^{B}
Two-stage countercurrent extraction pH 9.36, 1:10 SLR, 50 °C, 45/75 min	-	50.5 ± 1.16^{B}	1058 ± 13.9^{B}	930 ± 168.0^{B}
Conventional solvent extraction 60% ethanol, 1:10 SLR, 50 °C, 75 min	-	62.5 ± 1.27^{A}	1112 ± 4.1^{A}	3005 ± 480.1^{A}

*Predicted TPC from regression model. **Experimental TPC from triplicate extractions. Different lowercase letters in the same row indicate significant ($p < 0.05$) differences between the predicted and experimental TPC of the optimized single-stage AEP. Different uppercase letters in the same column indicate significant ($p < 0.05$) differences in TPC or antioxidant activities between extraction methods

In the countercurrent process, the nearly depleted insoluble fiber-rich fraction from the first extraction was re-extracted with fresh water, which increased the concentration gradient of the system and favored mass transfer and overall phenolic extractability even at a higher SLR. This strategy enriches the final extract with a concentrated solution of bioactive compounds while also reducing the volume of fresh water needed to propel diffusion.

2.3.6. Antioxidant activity of single-stage and two-stage countercurrent AEP grape pomace extracts

The *in vitro* antioxidant activities of the grape pomace extracts were explored to provide insight into their potential radical scavenging applications in food, pharmaceutical, or cosmetic applications. As shown in Table 2.2, grape pomace extracts with higher TPC also showed higher antioxidant activities by both ABTS and ORAC radical scavenging methods. Both antioxidant assays were evaluated in this study to provide a more representative characterization of the antioxidant activity of the grape pomace extracts since their mechanisms of action are different. The ABTS method is a spectrophotometric assay that measures the single-electron transfer (SET) from the polyphenolic compounds in the grape pomace extract to the ABTS free radical (Speisky et al., 2017). The ORAC method is a fluorometric assay that utilizes a hydrogen atom transfer (HAT) reaction to terminate free radical propagation. The ORAC method is also considered more biologically relevant than the ABTS method since it measures the decomposition of peroxyl radicals similar to those generated from protein and lipid oxidation (Speisky et al., 2017)

The extract from the two-stage countercurrent process was over three times more powerful at inhibiting the ABTS radical compared to the single-stage AEP extract and exhibited a similar magnitude compared to the conventional solvent extract. A similar trend was observed with the ORAC method, where a 70% increase in antioxidant potential was observed when moving from the single-stage AEP to the countercurrent AEP, although the conventional solvent extract presented a significantly higher antioxidant potential.

The positive correlation between TPC and antioxidant activity by ABTS and ORAC methods has been well reported for grape and other fruit extracts (Dudonné et al., 2019; Ky & Teissedre, 2015; Monagas et al., 2005), and our data agree with the magnitude of the antioxidant potential reported using both ABTS and ORAC methods. Ky & Teissedre (2015) compared the

antioxidant activity of red grape skin and seed extracts from aqueous and 70% ethanol extractions performed at 1:3.5 SLR, 50 °C, and 60 min. The antioxidant activity of the aqueous skin and seed extracts ranged from 668–2433 μmol Trolox/g dry weight using the ABTS method and from 1034–2231 μmol Trolox/g dry weight using the ORAC method. Ethanol extractions showed higher antioxidant capacities, similar to the present study, with an ABTS range of 1923–3601 μmol Trolox/g dry weight and an ORAC range of 1239-2614 μmol Trolox/g dry weight.

Despite these similarities, direct comparisons between analyses are complicated, since the TPC and antioxidant activity of grape pomace extracts can differ due to grape cultivar, climate, soil type, winery scale, drying and storage conditions, type of pomace material tested (individual or mixed components: skins, seeds, stems, pulp), and extraction conditions used. Winemaking methods can also affect grape pomace composition, and subsequently, the phenolic profile and biological activity of the extracts. For example, the Cabernet Sauvignon pomace used in this study was collected prior to fermentation, so high residual sugars were expected, which influences the concentrations and forms of the insoluble-bound phenolics and their activities. In addition, the grapes were pressed at low pressure (1.6 psi), which contributes not only to residual moisture in the grape skins, but also to residual color (i.e., anthocyanins), which are strongly correlated to both *in vivo* and *in vitro* antioxidant capacity (Lingua et al., 2015; Valls et al., 2017).

2.3.7. Phenolic composition of Cabernet Sauvignon grape pomace extracts

An untargeted metabolomics approach was used to screen for phenolic compounds in the single-stage AEP, two-stage countercurrent AEP, and hydroethanolic extracts of the unfermented Cabernet Sauvignon pomace. The untargeted method allows for a broader screening and identification of a more diversified pool of phenolics associated with unfermented Cabernet

Sauvignon grape skins and seeds. Overall, the hydroethanolic extracts from conventional solvent extraction presented the most diverse polyphenolic profile with 33 identified and annotated compounds followed by the extracts from the two-stage countercurrent AEP (18 identified and annotated compounds) and the single-stage AEP (15 identified and annotated compounds) (Figure 2.6). Single-stage and two-stage AEP extracts showed similar phenolic compositions and were mainly composed of hydroxycinnamic acids, hydroxybenzoic acids, and flavonols. There were fewer identifications of these classes in the hydroethanolic extract, which was richer in anthocyanins and flavanols (Figure 2.6). Further improvements in aqueous extraction methods are warranted to target the recovery of anthocyanins and flavan-3-ols, which can improve the antioxidant activities of grape pomace extracts and provide various human health benefits (Monrad et al., 2014).

In the single-stage AEP extracts, the compounds with the highest relative contents were isomers of trans-melilotoside followed by gentisic acid 5-O-glucoside, isorhamnetin 3-galactoside, and myricitrin (Supplementary Materials—Table S2.5). These compounds were also the major constituents of the extracts from two-stage countercurrent extraction (Figure 2.6). Melilotoside is a precursor of coumarin and is stored as a glucoside of trans-2-coumaric acid in plant vacuoles (Liu et al., 2010). In wine grapes, melilotoside can undergo innate enzymatic or photochemical isomerization to form coumaric acid and coumarin (Hroboňová & Sádecká, 2020). As secondary plant metabolites, coumarins have exhibited antibacterial, antifungal, anti-inflammatory, and antioxidant activities (Borges et al., 2005). Gentisic acid is one of the most prominent hydroxybenzoic acids in wine grapes and is associated with anticholinesterase activity (Stój et al., 2019). The presence of its glycosidic form in all three extraction treatments reflects the abundance of sugars in the extracts due to the grape pomace being collected prior to

fermentation, and an increase in the relative content of gentisic acid in the two-stage countercurrent process is likely due to the enrichment of the extracting media driving further diffusion. Isorhamnetin 3-galactoside, a derivative of quercetin, was more abundant in the countercurrent sample compared to the samples from single-stage AEP or conventional solvent extraction. The presence of these flavonols in red wine grapes has been further explored by Castillo-Muñoz et al. (2007) and Marchante et al. (2018). Myricitrin is a rhamnose glycoside of myricetin that has been associated with the biosynthesis of anthocyanins in grapes (Niu et al., 2016).

Unique to the two-stage countercurrent process compared to the single-stage AEP were the identification and annotation of p-coumaric acid, vanillic acid glucoside, and astilbin (Supplementary Materials—Table S2.5), which increased the presence of hydroxycinnamic and hydroxybenzoic acids in the countercurrent sample (Figure 2.6). Astilbin has been identified and quantified in Cabernet Sauvignon wine by (Landrault et al., 2002). The two-stage countercurrent process may have enhanced the structural degradation of the grape matrix and aided phenolic release by improving the solubilization of these additional compounds.

Naringenin 7-O-glucoside, also known as prunin, was identified in the hydroethanolic extract, but not in the AEP extracts (Supplementary Materials—Table S2.5). Naringenin is a major citrus flavanone displaying antioxidant and anti-inflammatory properties (Alam et al., 2014). It has been identified in Chardonnay white wines by Rocchetti et al. (2018) in a comprehensive study using untargeted metabolomics, and it was also reported in commercial dried Cabernet pomace (Ramirez-Lopez & DeWitt, 2014) and in Sercial and Tinta Negra grape skins (Perestrelo et al., 2012). Epicatechins, catechins, and procyanidins were also identified exclusively in the hydroethanolic extracts (Supplementary Materials—Table S2.5). The

identification of catechin isomers in the hydroethanolic extract may be due to the higher stability and better dissolution of these flavanols in ethanol compared to water due to the lower polarity and dielectric constant of ethanol (Cuevas-Valenzuela et al., 2014). While epicatechin gallate and catechin are more prominent in the grape skins, the procyanidin dimers and trimers are prevalent in grape seeds. The high relative content of procyanidin B2 in the hydroethanolic extract reflects previous results by Shi et al. (2003), where increasing ethanolic concentrations to 50% (v/v) improved extraction of procyanidin dimers from grape seed meal.

Other notable changes in phenolic composition across extraction treatments include the identification of malvidin 3,5-diglucoside in only the hydroethanolic extract, which may be due to better stability of anthocyanins at less alkaline conditions (Supplementary Materials—Table S2.5). Salviaflaside, a phenolic acid glycoside that is most notably recognized in Prunella vulgaris, was also identified in the grape pomace sample extracted with ethanol (Supplementary Materials—Table S2.5). This compound has not yet been widely reported in Cabernet Sauvignon grape pomace.

Trans-piceid, a glucoside of resveratrol, was found in all three treatments. Trans-piceid has been identified in red and white wines and can be converted to resveratrol by glucosidase activity in the intestine (Sato et al., 1997; Vian et al., 2005), which highlights the role of human digestion in transforming polyphenols into different functional forms. Secoisolariciresinol, the lignan commonly found in flaxseeds, was also identified in all three grape pomace extracts with an increase in its relative content in the countercurrent method. The presence of secoisolariciresinol in wine has also been reported by Dadáková et al. (2021). The liberation of this lignan from the grape cell-matrix in the present study is likely due to alkaline hydrolysis,

which has been widely used to release secoisolariciresinol from flaxseed complexes by breaking ester linkages (Fuentealba et al., 2015)

Overall, the use of untargeted profiling allowed for the discovery of compounds present in the unfermented Cabernet Sauvignon pomace that may have been otherwise overlooked by targeted approaches driven by a few select standard compounds. Notably, the use of red wine grape pomace sourced from a white winemaking process presents a unique scenario that allowed for the analysis of red wine abundant polyphenols (e.g., anthocyanins) alongside compounds that were not yet degraded by fermentation, as represented by the abundance of phenolic glycosides in the extracts like gentisic acid 5-O-glucoside and isorhamnetin 3-galactoside. The findings of this study support the valorization of red wine grape pomace for applications where diverse biological activity and functionality are desired, such as exploiting the antidiabetic properties of the phenolic-polysaccharide conjugates (Campos et al., 2021). While the current data highlights the impact of processing conditions on the extraction of selected phenolics, methods that provide absolute quantification of the major phenolic components must be employed to determine the exact concentrations in the sample.

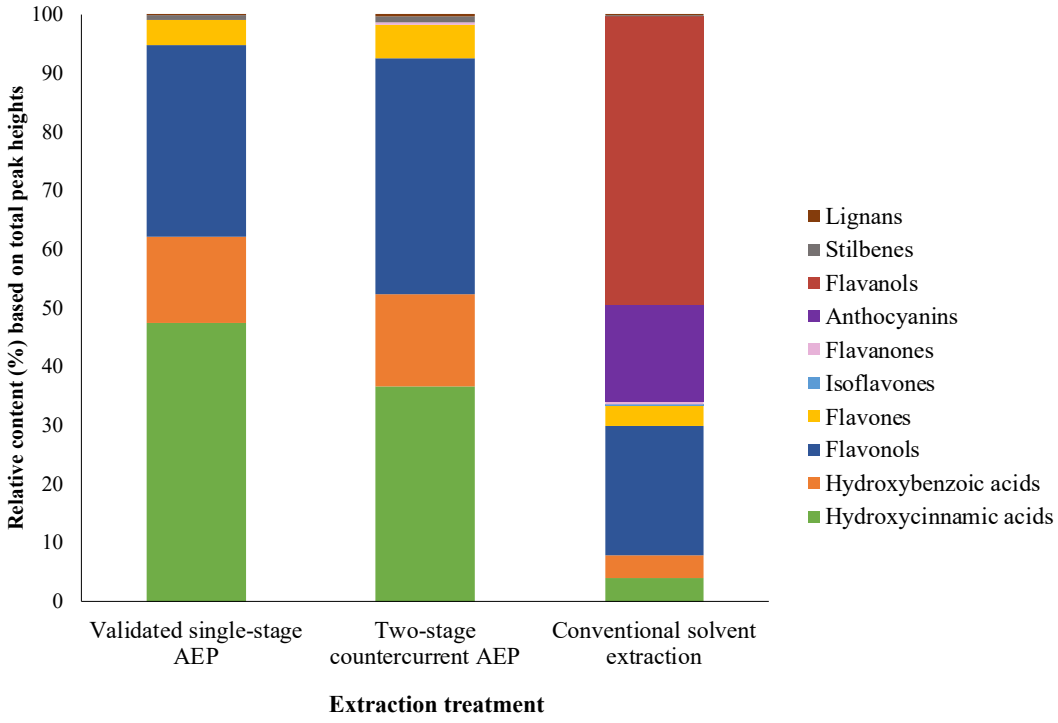


Figure 2.6. Phenolic composition of validated single-stage AEP, two-stage countercurrent AEP, and conventional solvent extracts as identified by untargeted metabolomics

2.4. Conclusions

An environmentally-friendly, two-stage countercurrent method for extracting phenolics from unfermented red wine grape pomace was successfully designed using alkaline conditions while reducing overall water consumption by 80%. The countercurrent extraction process enabled the extraction of phenolic acids, flavonols, and flavones from the grape cell-matrix with unique identifications of compounds that have not yet been widely associated with red wine grape skins and seeds. Until this study, there existed limited evidence of the effect of alkaline hydrolysis on enhancing phenolic release from unfermented Cabernet Sauvignon grape pomace. Additionally, this report highlights the role of untargeted metabolomics in elucidating the polyphenolic profile of the red wine grape pomace feedstock resulting from a white winemaking process. Overall, this study serves as a proof-of-concept for the use of a multi-stage

countercurrent extraction process at alkaline conditions to produce valuable, solvent-free, and antioxidant-rich extracts from underexplored winemaking waste streams. The benefits of alkaline conditions and the recirculation of processing streams in the countercurrent process were explicitly emphasized in this study, but the mechanism of phenolic release from the grape cell-matrix should be further explored to develop a deeper understanding of alkali-based extractions, the stability of the extracts, and the *in vivo* efficacy of products that incorporate the extracts as bioactive ingredients. Future research should also evaluate the economic feasibility of large-scale countercurrent designs at alkaline conditions to valorize winemaking byproducts and other agri-food waste streams for a variety of industrial applications.

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2.6. Supplementary Materials

Table S2.1. Variables and levels of the central composite rotatable design (CCRD) for the optimization of total phenolic content from wine grape pomace extracts

Variables	Levels				
	-1.68	**-1**	**0**	**+1**	**+1.68**
X_1: pH	2.64	4.00	6.00	8.00	9.36
X_2: SLR (g pomace/mL water)	1:50	1:17.7	1:9.1	1:6.1	1:5
X_3: Temperature (°C)	41.6	45.0	50.0	55.0	58.4

Table S2.2. Experimental variable levels and TPC responses of aqueous extractions from single-stage AEP optimization experiments

Experiment	Variable levels: Uncoded (Coded)			TPC (mg GAE/g dry grape pomace)					
	X_1 (pH)	X_2 (SLR)	X_3 (°C)	15 min	30 min	45 min	60 min	75 min	90 min
1	4.00 (-1)	1:17.7 (-1)	45.0 (-1)	7.77 ± 0.12 nopq	7.73 ± 0.15 mnopqr	8.01 ± 0.16 lmnop	8.67± 0.10 jkl	9.60± 0.37 fghi	9.36± 0.33 hij
2	8.00 (1)	1:17.7 (-1)	45.0 (-1)	21.51 ± 0.43 J	19.81 ± 0.43 K	22.13 ± 0.48 J	22.06 ± 0.10 J	24.24 ± 0.00 H	23.27 ± 0.06 I
3	4.00 (-1)	1:6.1 (1)	45.0 (-1)	5.90 ± 0.02 xyz!@#	5.83 ± 0.04 yz!@#	5.49 ± 0.25 @#	5.56 ± 0.04 !@#	6.55 ± 0.05 tuvwxy	7.04 ± 0.04 qrstu
4	8.00 (1)	1:6.1 (1)	45.0 (-1)	12.34 ± 0.13 TU	12.41 ± 0.25 STU	12.55 ± 0.10 ST	14.43 ± 0.02 Q	14.97 ± 0.05 PQ	15.31 ± 0.21 P
5	4.00 (-1)	1:17.7 (-1)	55.0 (1)	9.18 + 0.22 ijk	9.59 ± 0.14 fghi	10.68 ± 0.22 Yzabcd	10.74 ± 0.09 Yzabcd	11.12 ± 0.00 XYZa	12.59 ± 0.19 ST
6	8.00 (1)	1:17.7 (-1)	55.0 (1)	25.47 ± 0.24 G	25.23 ± 0.05 G	26.82 ± 0.05 F	28.16 ± 0.09 E	29.84 ± 0.41 D	32.09 ± 0.41 C
7	4.00 (-1)	1:6.1 (1)	55.0 (1)	5.69 ± 0.17 z!@#	6.03 ± 0.32 vwxyz!@	6.26 ± 0.07 uvwxyz!@	6.68 ± 0.19 stuvwx	6.47 ± 0.02 uvwxyz	7.49 ± 0.02 opqrs
8	8.00 (1)	1:6.1 (1)	55.0 (1)	12.12 ± 0.02 TUV	13.21 ± 0.02 RS	14.48 ± 0.13 Q	15.83 ± 0.05 OP	17.54 + 0.20 M	17.79 ± 0.16 LM
9	2.64 (-1.68)	1:9.1 (0)	50.0 (0)	7.69 ± 0.10 opqr	7.80 ± 0.03 nopq	8.01 ± 0.06 lmnop	8.64 ± 0.03 jklm	9.21 + 0.14 ijk	10.35 ± 0.03 abcdef
10	9.36 (1.68)	1:9.1 (0)	50.0 (0)	28.64 ± 0.03 E	29.91 ± 0.65 D	32.64 ± 1.37 C	33.89 ± 0.11 B	36.70 ± 0.51 A	36.76 ± 0.55 A
11	6.00 (0)	1:50.0 (-1.68)	50.0 (0)	13.40 ± 0.28 R	15.67 ± 0.48 P	17.66 ± 0.31 LM	16.53 ± 0.22 NO	17.04 ± 0.51 MN	18.44 ± 0.12 L
12	6.00 (0)	1:5.0 (1.68)	50.0 (0)	5.10 ± 0.03 #	5.96 ± 0.03 wxyz!@	6.32 ± 0.07 uvwxyz!@	6.49 ± 0.02 tuvwxyz	6.77 ± 0.03 stuvw	7.44 ± 0.04 opqrs
13	6.00 (0)	1:9.1 (0)	41.6 (-1.68)	6.95 ± 0.00 rstuv	7.28 ± 0.08 pqrst	7.28 ± 0.03 pqrst	7.74 ± 0.05 nopqr	8.17 ± 0.03 lmno	8.53 ± 0.05 klmn
14	6.00 (0)	1:9.1 (0)	58.4 (1.68)	9.34 ± 0.03 hijk	10.08 ± 0.33 fghi	9.78 ± 0.17 fghi	9.98 ± 0.03 defghi	10.18 ± 0.16 bcdefg	11.73 ± 0.24 UVWX
15	6.00 (0)	1:9.1 (0)	50.0 (0)	9.87 ± 0.13 efghi	10.72 ± 0.03 Yzabcd	11.05 ± 0.09 XYZa	11.32 ± 0.08 WXYZ	10.69 ± 0.03 Yzabcd	12.13 ± 0.10 TUVW
16	6.00 (0)	1:9.1 (0)	50.0 (0)	10.05 ± 0.03 cdefgh	10.64 ± 0.08 Zabcde	10.86 ± 0.00 Yzabc	10.92 ± 0.03 XYZab	11.02 ± 0.08 XYZa	11.47 ± 0.03 VWXY
17	6.00 (0)	1:9.1 (0)	50.0 (0)	9.44 ± 0.06 ghij	11.27 ± 0.10 XYZ	11.00 ± 0.10 XYZa	11.32 ± 0.16 WXYZ	11.15 ± 0.05 XYZa	11.37 ± 0.06 WXYZ

Data represent mean ± one standard deviation of triplicates. Different letters (uppercase and lowercase) across all treatments indicate significant ($p < 0.05$) differences among TPC of all extracts using two-way ANOVA

Table S2.3. ANOVA results of the regression model predicting TPC of single-stage AEP grape pomace extracts

Source	SS	df	MS	F_{calc}	F_{tab}	*p*-value
Regression	1157.7	5	231.5	94.3	3.2	< 0.0001
Residuals	27.0	11	2.5			
Lack of fit	26.9	9	3.0	53.1	19.4	0.0186
Pure error	0.1	2	0.1			
Total	1184.8	16				

Table S2.4. Predicted and experimental total phenolic content of optimization experiments at 75 min of extraction time

	Variable levels			Extraction time: 75 min		
Experiment	**X_1 (pH)**	**X_2 (SLR)**	**X_3 (°C)**	**Predicted TPC**	**Experimental TPC**	**% Error**
1	4.00	17.7	45.0	8.52	9.60 ± 0.37	12.68
2	8.00	17.7	45.0	26.50	24.24 ± 0.00	-8.53
3	4.00	6.1	45.0	5.16	6.55 ± 0.05	26.94
4	8.00	6.1	45.0	16.22	14.97 ± 0.05	-7.71
5	4.00	17.7	55.0	10.42	11.12 ± 0.00	6.72
6	8.00	17.7	55.0	28.40	29.84 ± 0.41	5.07
7	4.00	6.1	55.0	7.06	6.47 ± 0.02	-8.36
8	8.00	6.1	55.0	18.12	17.54 + 0.20	-3.20
9	2.64	9.1	50.0	10.74	9.21 + 0.14	-14.28
10	9.36	9.1	50.0	35.14	36.70 ± 0.51	4.45
11	6.00	50.0	50.0	16.45	17.04 ± 0.51	3.59
12	6.00	5.0	50.0	4.99	6.77 ± 0.03	35.64
13	6.00	9.1	41.6	9.12	8.17 ± 0.03	-10.46
14	6.00	9.1	58.4	12.32	10.18 ± 0.16	-17.34
15	6.00	9.1	50.0	10.72	10.69 ± 0.03	-0.28
16	6.00	9.1	50.0	10.72	11.02 ± 0.08	2.80
17	6.00	9.1	50.0	10.72	11.15 ± 0.05	4.01

Table S2.5. Relative abundance of phenolics in grape pomace extracts using an untargeted metabolomics approach

Polyphenol class	Compound	Single-stage AEP				Two-stage countercurrent AEP				Conventional solvent extraction			
		Avg. peak height*	SD	CV (%)	Relative content (%)	Avg. peak height*	SD	CV (%)	Relative content (%)	Avg. peak height*	SD	CV (%)	Relative content (%)
Phenolic acids													
Hydroxycinnamic acids	*p*-Coumaric acid					85156	3500	4	**0.67**				
	Salviaflaside									292694	35598	12	**0.34**
	trans-Melilotoside Isomer A	566237	15413	3	**16.63**	1744123	143062	8	**13.67**	2131568	195898	9	**2.48**
	trans-Melilotoside Isomer B	993268	30962	3	**29.17**	2681657	263736	10	**21.03**	1018940	86795	9	**1.18**
	trans-Melilotoside Isomer C	58996	3884	7	**1.73**	157099	14308	9	**1.23**				
Hydroxybenzoic acids	Gentisic acid 5-*O*-glucoside	493367	27893	6	**14.49**	2004059	192386	10	**15.71**	2836660	260940	9	**3.30**
	Vanillic acid glucoside					11356	2388	21	**0.09**	564054	82193	15	**0.66**
Flavanoids													
Flavonols	Kaempferol									82484	24014	29	**0.10**
	Kaempferol 3-alpha-L-arabinopyranoside									151258	44279	29	**0.18**
	Quercitrin	22801	1458	6	**0.67**	99440	5527	6	**0.78**	542963	98927	18	**0.63**
	Quercetin									988523	148047	15	**1.15**
	Quercetin 3-*O*-glucuronide	137739	7441	5	**4.05**	680274	45171	7	**5.33**	2586774	463941	18	**3.01**
	Quercetin 3,4'-diglucoside	22476	3504	16	**0.66**	116919	6295	5	**0.92**	367162	50788	14	**0.43**
	Isoquercitin	45196	4876	11	**1.33**	178972	17764	10	**1.40**	1381963	247925	18	**1.61**
	Isorhamnetin 3-galactoside	402431	24745	6	**11.82**	1897979	93813	5	**14.88**	5771506	689177	12	**6.71**
	Myricetin									53530	10408	19	**0.06**
	Myricitrin	400860	29513	7	**11.77**	1885177	148786	8	**14.78**	6161454	906455	15	**7.16**
	Rutoside	34612	4163	12	**1.02**	86598	12736	15	**0.68**	157076	10257	7	**0.18**
	Syringetin 3-glucoside	46864	4597	10	**1.38**	179294	23690	13	**1.41**	669266	98871	15	**0.78**
Flavones	Luteolin 7-glucoside	145858	2216	2	**4.28**	721022	55090	8	**5.65**	2017684	202634	10	**2.34**
	Tricin									922955	228075	25	**1.07**
	Jaceoside									122939	30108	24	**0.14**
Isoflavones	Iridin									79226	13632	17	**0.09**
Flavanones	Naringenin 7-*O*-glucoside									161384	10807	7	**0.19**
	Astilbin Isomer A					49091	4354	9	**0.38**	153440	30809	20	**0.18**
Anthocyanins	Malvidin 3,5-diglucoside									14200375	1448918	10	**16.50**
Flavanols	(-)-Epichatechin												
	Epicatechin monogallate									300457	36764	12	**0.35**
	Catechin Isomer A									106950	22744	21	**0.12**
	Catechin Isomer B									2420931	581237	24	**2.81**
	Catechin Isomer C									40347	3136	8	**0.05**
	Procyanidin B2 Isomer A									14382478	1064516	7	**16.71**
	Procyanidin B2 Isomer B									17969239	2586525	14	**20.88**
	Procyanidin C1									7177177	935123	13	**8.34**
Stilbenes	*trans*-Piceid	29440	531	2	**0.86**	117703	7046	6	**0.92**	110454	10585	10	**0.13**
Lignans	Secoisolariciresinol	4564	963	21	**0.13**	58478	1227	2	**0.46**	136653	14966	11	**0.16**
Sum of peak heights		**3404709**				**12754397**				**86060563**			

*Average peak heights are the result of triplicate samples

Table S2.6. Method parameters for untargeted phenolic profiling

Polyphenol class	Compound	Species	*m/z*	ESI mode	RT
Phenolic acids					
Hydroxycinnamic acids	*p*-Coumaric acid	[M+H]+	165.0546	positive	1.382
	Salviaflaside	[M-H]-	521.1303	negative	2.157
	trans-Melilotoside Isomer A	[M-H]-	325.0930	negative	0.526
	trans-Melilotoside Isomer B	[M-H]-	325.0931	negative	0.695
	trans-Melilotoside Isomer C	[M-H]-	325.0931	negative	1.184
Hydroxybenzoic acids	Gentisic acid 5-*O*-glucoside	[M-H]-	315.0724	negative	0.489
	Vanillic acid glucoside	[M-H]-	329.0880	negative	1.297
Flavanoids					
Flavonols	Kaempferol	[M+H]+	287.0551	positive	4.431
	Kaempferol 3-alpha-L-arabinopyranoside	[M+Na]+	441.0818	positive	2.535
	Quercitrin	[M+H]+	449.1079	positive	2.465
	Quercetin	[M-H]-	301.0356	negative	3.731
	Quercetin 3-*O*-glucuronide	[M+H]+	479.0823	positive	2.079
	Quercetin 3,4'-diglucoside	[M-H]-	625.1416	negative	1.503
	Isoquercitin	[M+H]+	465.1031	positive	2.111
	Isorhamnetin 3-galactoside	[M-H]-	477.1040	negative	2.563
	Myricetin	[M-H]-	317.0306	negative	2.893
	Myricitrin	[M+Cl]-_[M-H]-	499.0652_463.0886	negative	2.051
	Rutoside	[M-H]-	609.1471	negative	1.950
	Syringetin 3-glucoside	[M+Na]+	531.1110	positive	2.671
Flavones	Luteolin 7-glucoside	[M-H]-	447.0936	negative	2.415
	Tricin	[M+H]+	331.0806	positive	1.897
	Jaceoside	[M+H]+	331.0837	positive	3.856
Isoflavones	Iridin	[M+FA-H]-	567.1361	negative	2.521
Flavanones	Naringenin 7-*O*-glucoside	[M-H]-	433.1144	negative	2.683
	Astilbin Isomer A	[M-H]-	449.1093	negative	2.069
Anthocyanins	Malvidin 3,5-diglucoside	[Cat-2H-C6H8O4]-	509.1302	negative	1.572
Flavanols	(-)-Epichatechin	[M+Cl]-	325.0484	negative	1.224
	Epicatechin monogallate	[M-H]-	441.0831	negative	1.912
	Catechin Isomer A	[M+CHO2]-	335.0774	negative	0.969
	Catechin Isomer B	[M+H]+	291.0863	positive	1.044
	Catechin Isomer C	[M+H]+	291.0864	positive	0.555
	Procyanidin B2 Isomer A	[M-H]-	577.1353	negative	0.468
	Procyanidin B2 Isomer B	[M-H]-	577.1354	negative	0.626
	Procyanidin C1	[M-H]-	865.1991	negative	1.058
Stilbenes	*trans*-Piceid	[M-H]-	389.1245	negative	1.982
Lignans	Secoisolariciresinol	[M+FA-H]-	407.1713	negative	2.661

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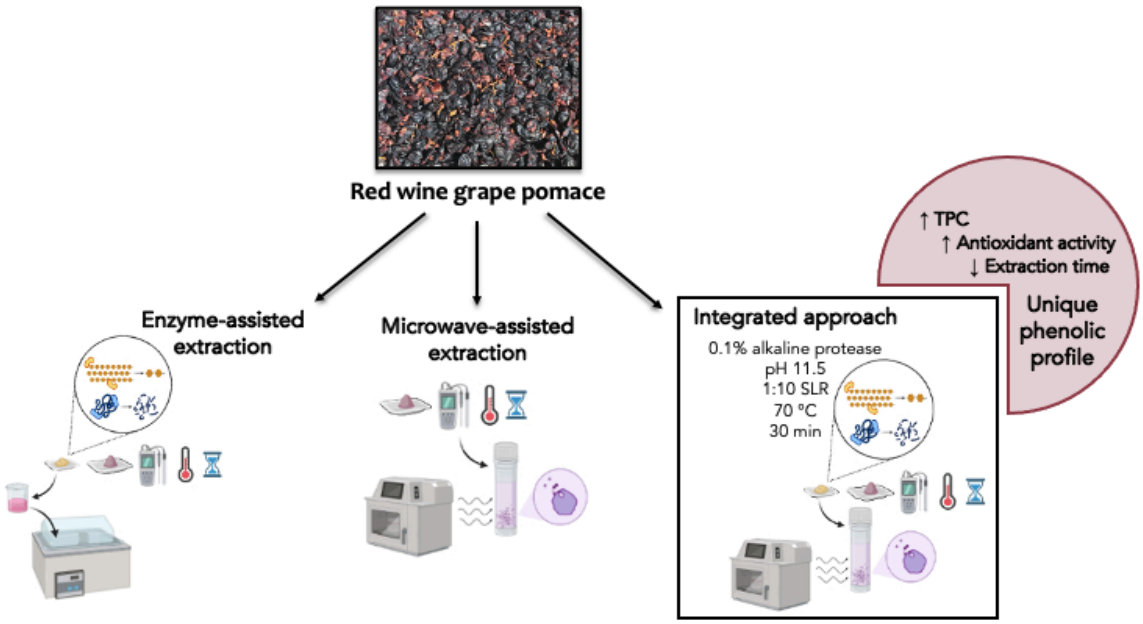
Chapter 3: Synergistic effects of proteolysis and microwave-assisted extraction on the recovery of bioactive phenolics from unfermented Cabernet Sauvignon pomace

Graphical abstract



Abstract

Enzyme-assisted (EAE), microwave-assisted (MAE), and microwave enzyme-assisted (MEAE) extractions were explored as greener alternatives to ethanol extraction methods (CSE, conventional solvent extraction, and MSE, microwave solvent extraction) for the extraction of phenolics from unfermented Cabernet Sauvignon grape pomace. To maximize the total phenolic content (TPC) of the extracts, the extraction time, temperature, slurry pH, solids-to-liquid ratio (SLR, g pomace/mL water), and type, combination, and concentration of enzymes(s) were selected by stepwise screening experiments. The overall TPC of the extracts were ranked as follows: MEAE (100.9 ± 2.09 mg GAE/g dry pomace) > MAE (91.3 ± 1.79 mg GAE/g dry pomace) > EAE (65.5 ± 3.24 mg GAE/g dry pomace) > AEP (58.8 ± 0.66 mg GAE/g dry pomace) > CSE (47.3 ± 1.78 mg GAE/g dry pomace) > MSE (44.6 ± 1.47 mg GAE/g dry

pomace). Overall, the addition of 0.1% FoodPro® alkaline protease in MEAE (1:10 SLR, initial pH of 11.5, 70 °C, 30 min) reduced the extraction time by 50% compared to AEP, EAE, and CSE methods and doubled the TPC of the extracts compared to CSE and MSE methods. The use of microwave processing, with or without enzymatic hydrolysis, also resulted in grape pomace extracts with *in vitro* antioxidant activities, as measured by ABTS and ORAC methods, that were similar to those of the hydroethanolic controls and greater than the AEP and EAE extracts. Untargeted metabolomics identified a higher relative content of catechins, procyanidins, trans-piceid, and malvidin-3,5-diglucoside in MEAE, MAE, MSE, and CSE samples compared to AEP and EAE extracts, which provides insight into the relationship between the phenolic composition and antioxidant activity of grape pomace extracts. Further, scanning electron microscopy (SEM) illustrated the synergistic effects of heat, pressure, and proteolysis during microwave processing that facilitated the disruption of the grape pomace cell wall structure and aided in the release of the flavonoid glycosides. These results serve as a proof-of-concept for the MEAE process as an effective strategy for generating bioactive ingredients for potential food, beverage, cosmetic, and pharmaceutical applications while achieving environmental sustainability goals.

Highlights

- MAE and MEAE improved phenolic extraction from grape pomace while reducing reaction time.
- Antioxidant activities of MAE and MEAE were similar to CSE extracts.
- Alkaline pH aided in phenolic release from the grape pomace, as identified by SEM.
- Phenolic compositions were influenced by alkaline protease and microwave radiation.

Keywords

Grape pomace; microwave-assisted extraction; enzyme-assisted extraction; alkaline pH; protease; phenolics

3.1. Introduction

The valorization of agricultural byproducts plays a critical role in maintaining environmental and economic sustainability at global scales. Addressing food loss and waste has evolved as a key objective for food producers, distributors, consumers, and legislative authorities who are passionate about reducing their environmental footprint while maintaining the profitability and longevity of the food system (United Nations Environment Programme, 2021). Intrinsic to the agri-food industry is a variety of waste mitigation strategies such as composting residual biomass or diverting it to livestock feed, but the inadequacies of these alternatives still leave abundant valuable components (carbohydrates, fibers, oils, proteins, polyphenols, and other phytonutrients) underutilized (Costa et al., 2020). To support a more circular food system, bioactive compounds recovered from processing byproducts should be re-integrated into industry pipelines as functional ingredients in value-added products. The biorefinery concept outlines a variety of techniques—such as extraction, fermentation, and anaerobic digestion—that utilize the full potential of waste streams by maximizing the number of innovative products generated from each processing fraction (Clark & Deswarte, 2015; Jin et al., 2018). Furthermore, the processing mechanisms used to obtain these value-added products should emphasize environmentally conscious technologies to better support a greener approach to food and agricultural supply chain challenges.

As one of the most cultivated specialty fruit crops in the world, wine grapes and their associated waste streams provide an opportunity for further exploration of green valorization methods. In the winemaking industry, byproducts are generated upon grape harvesting, destemming, crushing, pressing, and sedimentation (Spigno et al., 2017). Wine grape skins, seeds, and pulp are collectively known as grape marc or pomace, and together they compose

approximately 25% of the original mass of the grape harvest (Gómez-Brandón et al., 2019). Grape pomace is especially rich in biologically relevant compounds such as polyphenols, dietary fibers, oligosaccharides, proteins, fatty acids, vitamins, and minerals (Deng et al., 2011) that could be used as health-promoting ingredients for food, beverage, pharmaceutical, and nutraceutical applications (Antonić et al., 2020; Kalli et al., 2018).

Conventional extraction methods for obtaining bioactive compounds from complex grape pomace matrices typically rely on harsh organic solvents like methanol or more benign flammable solvents like ethanol, both of which require subsequent processing for downstream applications in food-grade products (Azmir et al., 2013). Not only do hazardous solvents pose human and ecological health and safety concerns, but they also increase the operational costs associated with large-scale commercialization due to the need for additional evaporative unit operations. Therefore, emerging research on green technologies has provided a wide variety of alternative methods that reduce or entirely avoid the use of organic solvents for the extraction of bioactives from grape pomace (Moro et al., 2021; Sirohi et al., 2020). These methods have the capability of improving phenolic extraction yields, reducing solvent use, and minimizing overall reaction time by targeting the degradation of the plant cell wall integrity and thus increasing the solubility and diffusion of intracellular compounds into the solvent for further recovery.

Especially favorable in the extraction of thermolabile phytochemicals is the application of the relatively mild heating conditions used in aqueous-, enzyme-, and microwave-assisted extractions. Careful selection of moderate extraction temperatures in the aqueous extraction process (AEP) can simultaneously protect sensitive phenolics like catechin, epicatechin, and resveratrol (Esparza et al., 2020) while also providing an opportunity to promote optimal enzyme activity upon their addition in the enzyme-assisted extraction (EAE) methods. In Chapter 2, it

was shown that a maximum TPC of unfermented Cabernet Sauvignon pomace was achieved using the two-stage countercurrent AEP at pH 9.36 using a 1:10 solids-to-liquid ratio at 50 °C for 75 min. However, total phenolic extractability could be improved with the use of enzymes in the single-stage AEP or two-stage countercurrent methods. Since grape skin cell walls are estimated to contain about 30% neutral polysaccharides (cellulose, xyloglucan, arabinan, galactan, xylan, and mannan), 20% acidic pectin compounds, 15% insoluble proanthocyanidins, and 5% structural proteins (Chamorro et al., 2012; Lecas & Brillouet, 1994), enzymes like cellulase, hemicellulase, glucanase, pectinase, and protease are often employed to aid in phenolic release. Different classes of enzymes have also been applied in combination to evaluate the effect of various hydrolytic mechanisms on extraction yields (Shen et al., 2021).

Microwave-assisted extraction (MAE) can complement both aqueous- and enzyme-assisted extractions to further degrade cell matrices and reduce extraction time through induced dipole rotation. Exposure of the polar aqueous solvent to microwave irradiation creates vibrations among water molecules as they are directed by the frequency of the microwave field. The resulting oscillation of water molecules within the grape cell matrix generates friction, which creates heat within the system and propels mass transfer (Bagade & Patil, 2019). This phenomenon enables rapid extraction of solutes as the surges in temperature and pressure eventually cause the plant cell to rupture.

In AEP, EAE, and MAE, phenolic extraction is influenced by solvent nature, sample composition and particle size, and reaction parameters like temperature and time. In green extractions using water as the only solvent, the pH of the slurry plays a key role in determining the type and stability of compounds extracted. Although some anthocyanins are typically more stable in acidic conditions, other compounds like ferulic acid, (-)-catechin, (-)-epigallocatechin,

rutin, and trans-cinnamic acid have shown stability in alkalinity up to pH 11 (Friedman & Jürgens, 2000). Exposure to alkaline treatments is effective at liberating bound phenolics from protein and carbohydrate complexes by cleaving both ether and ester bonds (Acosta-Estrada et al., 2014; Shahidi & Yeo, 2016), but few studies have evaluated the effect of a range of pH on phenolic extraction using water as the solvent. In Chapter 2, it was reported that increasing the aqueous slurry pH to 9.36 resulted in maximum phenolic yields from unfermented Cabernet Sauvignon pomace, but further opportunities are available to enhance phenolic extractability using enzymes. Similarly, Librán et al. (2013) reported significant increases in total flavonols, flavanols, phenolic acids, and anthocyanins at pH 12 compared to pH 2, 5.33, 8.66 in water-based grape pomace extracts. However, the effect of extraction parameters on phenolic recovery from grape pomace using an integrated microwave enzyme-assisted extraction process has yet to be thoroughly conceptualized.

Therefore, the overall goal of this study was to explore the effect of fundamental extraction parameters on the total phenolic content, antioxidant activity, and phenolic profile of unfermented Cabernet Sauvignon pomace during enzyme-assisted, microwave-assisted, and microwave enzyme-assisted extractions. Specifically, the extraction time, temperature, slurry pH, solids-to-liquid ratio, and type and concentration of enzyme(s) were examined to maximize phenolic extractability using the aforementioned green extraction treatments. Scanning electron microscopy was also used as a tool to provide a mechanistic understanding of the impact of the extraction methods evaluated, alone or in combination, on the overall phenolic extractability and structure, phenolic composition by untargeted metabolomics, and the *in vitro* biological activities of the extracts as evaluated by ABTS and ORAC. The results of this study will provide guidance

for developing and commercializing alternative extraction methods to promote flammable solvent-free solutions for the agri-food industry.

3.2. Materials and Methods

3.2.1. Chemicals and reagents

Folin–Ciocalteu phenol reagent, Trolox®, potassium persulfate, 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH radical), and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS radical), D(+)-glucose, sulfuric acid, phenol, and fluorescein were supplied by Sigma-Aldrich (Saint Louis, MO, USA). Anhydrous gallic acid was purchased from Chem-Impex International (Wood Dale, IL, USA). Phosphate buffer solution was obtained from bioWORLD (Dublin, OH, USA). Anhydrous sodium carbonate was purchased from VWR Chemicals, BDH® (Solon, OH, USA). Ethanol (95% v/v) was supplied by Decon Labs (King of Prussia, PA, USA).

The following commercial enzymes were used in the enzyme screening process: pectinase was supplied by TCI (Tokyo, Japan) as a powdered enzyme derived from Aspergillus niger, and FoodPro® BSL, CBL, and Alkaline Protease were provided by the Genecor Division of DuPont™ Danisco® (Rochester, NY, USA). FoodPro® BSL is a liquid cellulase preparation derived from Trichoderma reesei. FoodPro® CBL is a liquid enzyme complex derived from Trichoderma reesei that hydrolyzes beta-glucans and non-starch polysaccharides like arabinoxylans. FoodPro® Alkaline Protease is a liquid endoprotease derived from Bacillus licheniformis.

3.2.2. Grape pomace preparation and composition

Red wine grapes (*Vitis vinifera* L. cv. Cabernet Sauvignon) were harvested from the student rotation block of the University of California, Davis Robert Mondavi Institute vineyard

(approximate latitude: 38.532 and longitude: -121.753) in October 2019. The grapes were stored at -16 °C until January 2020 when production of a white Cabernet Sauvignon wine began. The grapes were thawed, crushed, destemmed, and pressed (Bucher Xplus 22, Bucher Vaslin North America, Santa Rosa, CA, USA) up to 1.6 psi to avoid excessive color extraction. The juice was subsequently racked and fermented for white wine production, while the residual skins, seeds, pulp, and stems were stored at -20 °C. Aliquots of frozen sample were then gathered, and the grape stems were manually separated from the seeds, skins, and pulp. The seeds, skins, and pulp (called "pomace") was blended for 10 minutes in a Vitamix 5200 blender (Vitamix, Cleveland, Ohio, USA) in batches of approximately four cups at a time to homogenize the mixture. Prior to extraction, aliquots of the bulk frozen pomace were defrosted for 20 minutes at room temperature and ground to a fine paste using a mortar and pestle for approximately 5 minutes.

Proximate composition of the grape pomace was determined using standard AOAC and AOCS methods outlined in Chapter 2. The grape pomace contained 57.8 ± 0.09% moisture, 2.86 ± 0.07% protein, 1.87 ± 0.02% ash, 1.03 ± 0.09% lipids, and 36.4% carbohydrates (measured by difference). The moisture content of the grape pomace was used to determine the dry weight representations of the chemical analyses (TPC, ABTS, and ORAC methods).

3.2.3. Extraction methods

3.2.3.1. Screening of enzyme type and combination for enzyme-assisted extraction (EAE)

The effect of enzymatic hydrolysis on the release of phenolic compounds was evaluated using a stepwise optimization approach. First, an enzyme screening was performed to evaluate the effect of enzymes with varied specificities on the total phenolic content of the extracts. The series of experiments for the enzyme screening procedure are outlined in Figure 3.1. Three carbohydrases (pectinase, FoodPro® BSL, and FoodPro® CBL) and an alkaline protease were

tested both individually and in tandem. For each experiment, the solids-to-liquid ratio and temperature were held constant at 1:50 SLR and 50 °C. Extractions using carbohydrases were performed at pH 5.0 based on conditions provided by the manufacturers for the optimal activity of the enzymes. Extractions using FoodPro® alkaline protease were performed at pH 9.36 based on the conditions provided by the manufacturer and from the results from the aqueous extraction optimization previously performed in Chapter 2. The slurry pH was adjusted using 0.5–1 M NaOH or 0.5 M HCl added dropwise (< 2 mL) and maintained throughout extraction every 10 min. The extraction time was dependent on whether a carbohydrase or protease was used individually (75 min) or if a carbohydrase pre-treatment was used prior to the protease-assisted extraction (30 min pre-treatment + 75 min extraction). The selection of the 30 min pre-treatment time was guided by previous research on enzyme-assisted extraction by Souza Almeida et al. (2021), and the preliminary 75 min extraction time was based on previous research (Chapter 2) that optimized the parameters of the AEP. The total extraction time will be further explored in this study at a later iteration.

All enzyme-assisted extractions (EAE) employed an enzyme dosage of 0.5% carbohydrase and/or 0.5% alkaline protease (weight of enzyme/weight of wet grape pomace sample). Each enzymatic extraction was performed in triplicate in a temperature-controlled water bath using a magnetic stir plate (Cimarec™ i Telesystem Multipoint Stirrers, Thermo Scientific, Waltham, MA, USA). After extraction, the extracts were separated by centrifugation and filtration (Whatman Grade 1 filter papers, Sigma-Aldrich, Saint Louis, MO, USA).

For each enzymatic extraction condition, three control extractions were performed at the same extraction conditions above described, except for the lack of enzyme use, to understand the

effect of the extraction parameters on phenolic extractability. All extracts were stored at -20 °C until further analysis.

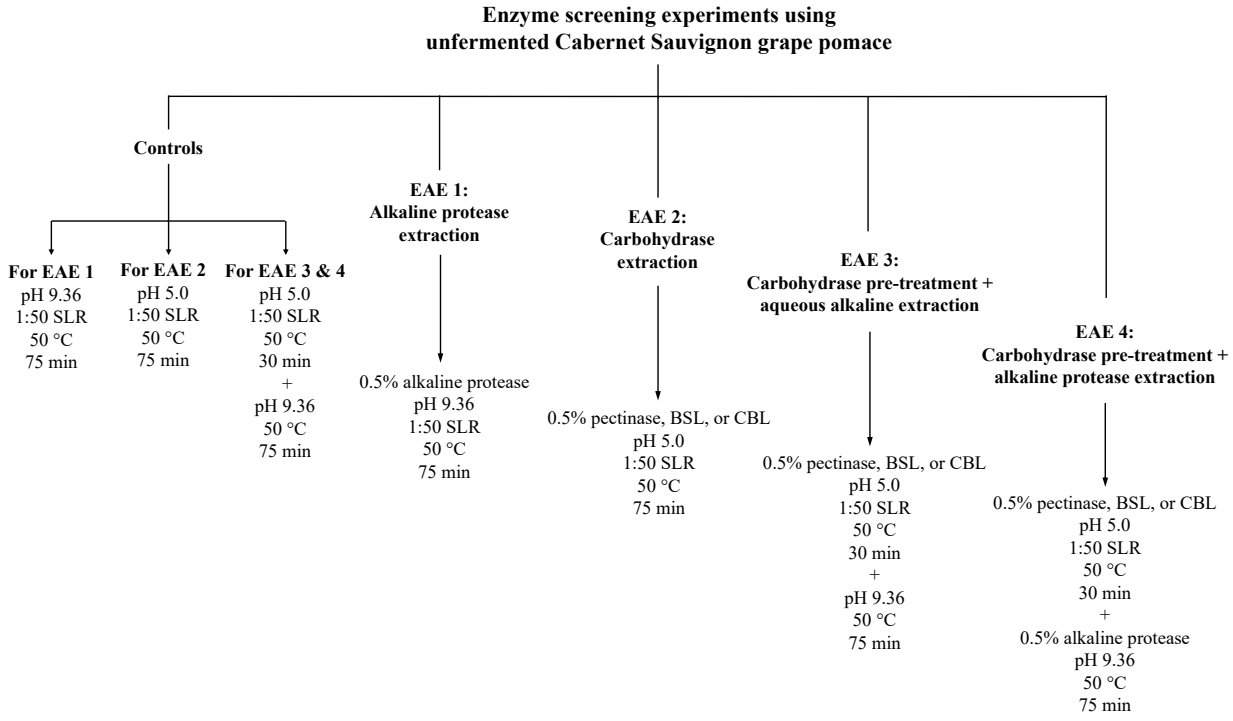


Figure 3.1. Schematic detailing of the enzyme-assisted extraction (EAE) screening experiments for the extraction of phenolics from unfermented Cabernet Sauvignon grape pomace

3.2.3.2. Selection of enzyme-assisted extraction (EAE) parameters

Based on the TPC results from the enzyme screening step (Section 2.3.1), another iterative screening process was used to select the enzyme dosage, extraction time, solids-to-liquid ratio (SLR), and slurry pH that best induced phenolic extraction from the grape pomace. Two separate investigations were performed: one to evaluate the effect of FoodPro® alkaline protease alone, and another to evaluate the use of a pectinase pre-treatment prior to the alkaline protease extraction. The use of 0.1, 0.5, 1.0, and 1.5% (w/w) alkaline protease was evaluated at pH 9.36, 1:50 SLR, 50 °C, and 75 min. Separately, the use of a 30-minute pectinase pre-

treatment was evaluated at an application of 0.5, 1.5, and 2.5% (w/w) followed by a 75-minute alkaline protease extraction at the selected protease concentration of 0.1% (w/w).

A kinetic study was then performed using the selected enzyme dosage at pH 9.36 with a 1:50 SLR (g pomace/mL water) at 50 °C. Aliquots were collected at 15, 30, 45, 60, and 75 min and immediately placed in an ice bath to prevent further extraction. Extraction kinetics were represented by the total phenolic content at each time point. Next, the SLR was evaluated at 1:10, 1:25, and 1:50 (g pomace/mL water) to determine the SLR leading to higher phenolic extraction yields. Extraction parameters were held constant at pH 9.36 with 0.1% alkaline protease (w/w) at 50 °C for the selected reaction time of 60 min. Lastly, since FoodPro® alkaline protease is stable up to pH 10.5, the pH of the grape pomace slurry was adjusted to pH 10.0 and pH 10.5 to explore how a solvent with greater alkalinity could affect phenolic extraction.

Each sequential extraction of the EAE stepwise screening process was performed in triplicate. Control experiments, at the optimal selected conditions, were also performed without including the enzyme. After extraction, all extracts were separated by centrifugation then filtered (Whatman Grade 1 filter papers, Sigma-Aldrich, Saint Louis, MO, USA) and stored at -20 °C until further analysis.

3.2.3.3. Selection of microwave-assisted extraction (MAE) parameters

The effects of extraction pH, SLR, temperature, and time were evaluated in the CEM MARS 6™ Microwave Digestion and Extraction System (CEM Corporation, Matthews, NC, USA) with 20 mL glass extraction vessels (GlassChem, CEM Corporation, Matthews, NC, USA) using a stepwise optimization approach. The microwave consists of a power system (0–1800 W), a magnetron (2450 MHz), and a Teflon®-coated microwave cavity holding the vessel turntable. The turntable fits up to 24 vessels, operates in alternating or continuous modes, and includes

magnetic stirring for each vessel with the addition of stir bars. The glass vessels were sealed with Teflon® PFA caps and nested in composite sleeves within the turntable. Sample temperatures were measured in real-time using a fiber optic temperature probe (MTS-300, CEM Corporation, Matthews, NC, USA) inserted in a control vessel filled with either water or ethanol depending on the treatment studied.

In the first step, the pH of the extraction slurry was adjusted to pH 9.36, 10.0, 10.5, 11.0, 11.5, and 12.0 while keeping the SLR, temperature, and time constant at 1:10 SLR (g pomace/mL water), 50 °C, and 60 min, respectively. An extraction time of 60 min was selected for the first round of MAE experiments based on the optimal results from the EAE. In the microwave, a 60-min extraction was accomplished by combining a ramp-up period of 5 min (time to reach the desired temperature) followed by a holding period of 55 min (time to held at desired temperature).

The pH range explored in MAE (pH 9.36–12.0) was selected based on observations from preliminary microwave-assisted extractions (preliminary data not shown). During MAE, the release of acidic compounds (e.g., phenolic acids) quickly decreases the slurry pH, but the target pH cannot be maintained due to the inaccessibility of the sample vessel while in the microwave system. Therefore, for screening experiments in the microwave, the pH was adjusted to alkaline conditions beyond the desired range to compensate for the drop in pH while still satisfying the conditions for stable activity of the FoodPro® alkaline protease. The pH of the extracts were measured before and after each extraction.

In the next step, various SLR (1:10, 1:25, and 1:50 g pomace/mL water) were evaluated with the volume of water held constant at 10 mL and the mass of grape pomace adjusted to achieve each targeted ratio. Each extraction was performed at pH 11.5 and 50 °C for 60 min.

Next, the extraction temperature was evaluated at 40, 50, 60, and 70 °C while the other extraction parameters were held constant at pH 11.5, 1:10 SLR, and 60 min. This temperature range includes conditions that are stable for both FoodPro® alkaline protease and many phenolic compounds. The microwave was programmed to fluctuate power as needed up to a maximum of 600 W to maintain the set temperature. Lastly, a kinetic study was performed to determine if extraction yields could be maintained while reducing total extraction time. The extraction times (5, 15, 30, 45, and 60 min) consisted of a 5 min ramp-up period with the remainder consisting of the holding time. All extracts in the microwave also completed a 10-min cooling cycle after each extraction, but this time was not included in calculating the total extraction time.

All extractions in each sequential step of the MAE screening process were performed in triplicate. After extraction, the extracts were separated by centrifugation and filtration (Whatman Grade 1 filter papers, Sigma-Aldrich, Saint Louis, MO, USA). All extracts were stored at -20 °C until further analysis.

3.2.3.4. Integrated microwave enzyme-assisted extraction (MEAE)

The integration of EAE and MAE was performed to determine if there were synergistic effects on the total phenolic content of grape pomace extracts. To optimize the integration, two variables were evaluated: i) the concentration (%) of alkaline protease, since the mechanism of enzyme activation may differ in the microwave and therefore may require a different dosage; and ii) the time of extraction, since enzyme kinetics and phenolic solubilization and diffusion to the extraction medium may differ when exposed to microwave irradiation in MEAE compared to conductive and convective heating in EAE.

To select the concentration of FoodPro® alkaline protease, extraction conditions were fixed at pH 11.5, 1:10 SLR, 70 °C, and 45 min (5 min ramp time + 40 min holding time). The

experiments were evaluated at 0.1, 0.5, 1.0, 1.5, 2.0, and 2.5% alkaline protease (weight of enzyme/weight of pomace) along with a control containing no enzyme. In the second step, total extraction time was tested at 5, 15, 30, 45, and 60 min using 0.1% alkaline protease (w/w) at pH 11.5, 1:10 SLR, and 70 °C. All MEAE extractions were performed in triplicate, and the final centrifuged and filtered extracts were stored at -20 °C until further analysis.

3.2.3.5. Conventional and microwave-assisted solvent extractions using ethanol

To compare the results of the EAE, MAE, and MEAE methods to their conventional solvent controls, a hydroethanolic mixture of 60% ethanol (v/v) was used as the solvent. Ethanol extractions were performed at a 1:10 SLR (g pomace/mL ethanol) without further pH adjustment. In the temperature-controlled water bath, as a control for EAE, the grape pomace slurry was agitated at 500 rpm for 60 min at 50 °C on a magnetic stir plate (Cimarec™ i Telesystem Multipoint Stirrers, Thermo Scientific, Waltham, MA, USA). Controls for MAE and MEAE were performed at 70 °C for 30 min (5 min ramp time + 25 min holding time) with agitation. All ethanol extractions were performed in triplicate followed by centrifugation, filtration, and storage at -20 °C.

3.2.4. Analysis of grape pomace extracts from EAE, MAE, and MEAE

1.1.1. Total phenolic content (TPC)

The total phenolic content of the extracts was determined according to the Folin-Ciocalteu spectrophotometric method as described by Singleton et al. (1999). Grape pomace extracts were diluted to read within the absorbance range of a gallic acid standard curve. In a clear 96-well microplate, 25 µL of the diluted sample was mixed with 125 µL of Folin-Ciocalteu:water solution (diluted 1:10, v/v, in nanopure water) at 300 rpm for 5 minutes at 37 °C. A sample of 100 uL of 7.5% (w/v) anhydrous sodium carbonate solution was added and agitated

again for 30 min then kept at rest for 90 min. The absorbance was read at 760 nm using a microplate reader (SpectraMax iD5, Molecular Devices, San Jose, CA). Results for each treatment (n = 9) were calculated using a standard curve of anhydrous gallic acid ($R^2 = 0.999$) with known concentrations of 5–95 µg/mL. TPC was expressed as mg of gallic acid equivalent (GAE)/g dry grape pomace extract.

1.1.2. ABTS and ORAC assays for antioxidant capacity

The ABTS assay, also known as the Trolox equivalent antioxidant capacity (TEAC) assay, was performed using the method of Al-Duais et al. (2009). The radical stock solution was produced by mixing 38.4 mg ABTS and 6.62 mg potassium persulfate in 10 mL deionized water. After incubating overnight in the dark, the radical stock solution was diluted with 95% (v/v) ethanol to obtain an initial absorbance of 0.70 ± 0.20 at 730 nm. Into a clear 96-well microplate, 20 µL of each grape extract sample diluted in ethanol was mixed with 200 µL diluted $ABTS^{\bullet+}$ solution. The mixture was agitated at 300 rpm for 6 min followed by reading at 730 nm using a spectrophotometer (SpectraMax iD5; Molecular Devices, San Jose, CA, USA). A calibration curve ($R^2 = 0.98$) using Trolox standard solutions (80–340 µM, diluted in ethanol) was used to calculate the results as µmol Trolox equivalent (TE)/g dry pomace.

The ORAC assay was performed according to procedures described by Zulueta et al. (2009). Briefly, 50 µL of fluorescein (78 nM) was mixed with 50 µL of grape pomace sample (diluted 1:2000, v/v, in phosphate buffer solution), blank (phosphate buffer solution), or standard (Trolox, 20 µM). An aliquot of 25 µL of 221 mM AAPH radical solution was added, and the microplate was read at 37 °C with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The fluorescence measurements were read every 5 min until the relative fluorescence intensity was less than 5% of the value of the initial reading. The ORAC values

were expressed as μmol Trolox equivalent (TE)/g dried pomace by determining μM Trolox using Equation 3.1, where C_{Trolox} represents the concentration of the standard (20 μM), DF is the sample dilution factor (2000), and AUC is the area below the fluorescence decay curve of the sample, blank, or Trolox.

$$\mu M\ Trolox = \frac{C_{Trolox}(AUC_{sample} - AUC_{blank}) * DF}{AUC_{Trolox} - AUC_{blank}} \quad \text{(Equation 3.1)}$$

1.1.3. Phenolic profiling by untargeted metabolomics

Identification of phenolics in triplicate grape pomace extracts was performed by the West Coast Metabolomics Center Central Services Core (UC Davis, Davis, CA, https://metabolomics.ucdavis.edu/core-services/assays-and-services) using a Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) using a PFP column. Phenolic identification and data processing was performed as described by Fiehn et al. (2008). A series of internal standards were added to the samples: Caffeine-d9, CUDA, daidzein-d4, genistein-d4, trans-cinnamic acid-d5, and hippuric acid-d5. A concentration of 0.5 μg/mL of hippuric acid-d5 was used with all other standards concentrated at 1 μg/mL in resuspension mix. Samples were resuspended to 100 μL with 0.5 μL injection volume for ESI positive mode and 5 μL for ESI negative mode (Supplementary Materials—Table S3.2). Compounds were identified by retention time and mass spectrum by comparing to established libraries. From the set of all identified and annotated compounds, only the annotated phenolic compounds were selected, sorted into classes, and reported. The reported peak heights were determined by dividing each metabolite peak by the sum of all peak heights for all identified metabolites for each sample.

1.1.4. Grape cell structure by scanning electron microscopy (SEM)

To investigate the effects of the various green extraction treatments on the grape pomace surface morphology, the insoluble fractions (residue from each extraction) of EAE, MAE, MEAE, and their control samples were evaluated using SEM. A sample of untreated, ground grape pomace sample was also analyzed to represent the starting material. Grape pomace tissues were rinsed twice in 0.1 M NaH_2PO_4 for 15 min each then dehydrated in an increasing series of ethanol (30–100%) and subjected to critical point drying (931.GL Supercritical Autosamdri, Tousimis Research Corporation, Rockville, MD, USA). The samples were mounted onto aluminum stubs and sputter-coated with gold (Pelco Auto Sputter Coater SC-7, Ted Pella, Inc., Redding, CA, USA) before imaging at 5 kV with magnification between 1000–1500× (Quattro S Environmental SEM, Thermo Scientific, Waltham, MA, USA).

3.2.5. Statistical analysis

All extractions and biological assays were performed in at least triplicate with the results expressed as mean ± standard deviation (SD) of the replicates. Analysis of variance (ANOVA) followed by the Tukey HSD test was performed using the Astatsa (2016, Navendu Vasavada) online program and JMP® (Trial 16.1.0, serial number T-TYPQDH0JJC, SAS Institute Inc., Cary, NC, USA) to determine significant differences among treatments at $p < 0.05$.

3.3. Results and Discussion

3.3.1. Effect of different enzymes and their combinations on phenolic extractability from grape pomace

It is established that the use of carbohydrases can aid in the release of polyphenols from plant cell walls, while proteases can cleave bound phenolics from structural proteins within the cellular matrix (Puri et al., 2012). The effects of various enzymatic treatments : (i) carbohydrase-assisted extraction (pectinase, FoodPro® BSL, or FoodPro® CBL); (ii) FoodPro® alkaline

protease-assisted extraction; (iii) carbohydrase pre-treatment followed by an alkaline aqueous extraction; and (iv) carbohydrase pre-treatment followed by alkaline protease-assisted extraction are shown in Figure 3.2A.

The phenolic yields across all enzyme screening experiments are shown in Figure 3.2A. Overall, the maximum TPC of 39.2 ± 1.66 mg GAE/g dry pomace resulted from the 30-min pre-treatment using 0.5% (w/w) pectinase followed by a 75-min extraction using 0.5% (w/w) FoodPro® alkaline protease. This phenolic yield is within the range of previous reports of EAE using wine grape pomace, where the phenolic yields ranged from 19.3 to 25.5 mg GAE/g dry pomace for mixed grape samples, 40.5 to 45.4 mg GAE/g dry pomace for white wine samples, and 51.8 to 60.1 mg GAE/g dry pomace for red wine samples using combinations of tannase, pectinase, and cellulase at 5% (w/w) total loading (Martins et al., 2016).

The other carbohydrase pre-treatments (FoodPro® BSL and CBL) followed by protease extraction also achieved high phenolic extractability (38.4 ± 0.45 and 37.0 ± 0.60 mg GAE/g dry pomace, respectively), being significantly higher than the TPC of the respective control experiment without enzymes at same pH conditions (pH 5.0 followed by pH 9.36) (TPC of 31.6 ± 0.75 mg GAE/g dry pomace). These results emphasize the action of the carbohydrases in hydrolyzing grape cell wall components and improving accessibility of the protease to structural proteins within the cell wall for the release of bound phenolics. However, all experiments conducted only at pH 5.0, regardless of carbohydrase use, produced the lowest phenolic contents with a TPC between 15.6–17.6 mg GAE/g dry pomace (Figure 3.2A). This finding agrees with the results described in Chapter 2, which emphasize the limited effects of acidic pH on the aqueous extraction of phenolics from the unfermented Cabernet Sauvignon pomace. Notably, there was no difference in the TPC of the alkaline control extraction without enzymes at pH 9.36 (31.9 ± 1.14

mg GAE/g dry pomace) compared to any of the carbohydrase pre-treatments at pH 5.0 followed by the shift to pH 9.36 (30.6–32.7 mg GAE/g dry pomace). Overall, the exposure to alkaline conditions alone was the main driver of phenolic extraction (TPC of 31.9 ± 1.14 mg GAE/g dry pomace), which was further enhanced by the addition of protease alone at alkaline conditions (TPC of 36.3 ± 1.11 mg GAE/g dry pomace), or the use of carbohydrases followed by protease at alkaline pH (TPC of 37.0 ± 0.60 to 39.2± 1.66 mg GAE/g dry pomace).

Importantly, the use of 0.5% (w/w) FoodPro® alkaline protease at pH 9.36 produced a TPC of 36.3 ± 1.11 mg GAE/g dry pomace after 75 min, which is only 8% lower than the TPC of the extracts obtained with the additional 30-min pectinase pre-treatment (Figure 3.2A). Our results agree with the literature, where the influence of high pH and protease-assisted treatments on promoting phenolic extractability from raspberry pomace press-cake has been reported by Saad et al. (2019). The simultaneous recovery of polyphenols and oils from raspberry pomace press-cake was best induced at pH 9 using 1.2% thermostable alkaline protease for 2 h at 60 °C, while the combination of alkaline protease followed by either pectinase and cellulase or by xylanase did not enhance polyphenolic yields or antioxidant activity compared to the application of protease alone (Saad et al., 2019). It was hypothesized that interferences from lignin may have obstructed the accessibility of the carbohydrases to their substrates, resulting in negligible improvements in phenolic extraction (Saad et al., 2019). Therefore, the costs associated with the prolonged extraction time, additional enzyme use, and intensified energy requirements of a pectinase pre-treatment process should be considered when evaluating the economic and environmental feasibility of EAE for industrial scale.

For this reason, the two best extraction scenarios (alkaline protease alone and alkaline protease preceded by pectinase pre-treatment) were more extensively evaluated to validate the

need for the pectinase pre-treatment and to explore the effect of their corresponding enzyme dosages on phenolic yields. As shown in Figure 3.2B, the use of 0.1% (w/w) FoodPro® alkaline protease significantly increased phenolic yields (35.0 ± 1.04 mg GAE/g dry pomace) compared to the aqueous control at pH 9.36 (31.9 ± 1.14 mg GAE/g dry pomace) and was not significantly different from the ones obtained when using 0.5% (36.3 ± 1.11 mg GAE/g dry pomace) and 1.0% (35.0 ± 1.32 mg GAE/g dry pomace) of protease. Although a slightly higher TPC (37.1 ± 0.42 mg GAE/g dry pomace) was produced using 1.5% (w/w) protease, the processing costs associated with the high enzyme application at a large scale would be counterproductive to justify the small increment in phenolic extraction. Therefore, 0.1% protease was selected as the fixed condition in the experiments exploring a pectinase pre-treatment loading of 0.5–2.5% (w/w).

Compared to the TPC of the extract obtained using only 0.1% (w/w) alkaline protease, no significant improvement was observed by the addition of an upstream 0.5% or 1.5% (w/w) pectinase pre-treatment (Figure 3.2B). Interestingly, the use of a 2.5% (w/w) pectinase pre-treatment did not increase phenolic extraction compared to the control without enzymes. This phenomenon is potentially due to an increase in slurry viscosity caused by an excessive addition of the powdered enzyme, which may have reduced mass transfer of phenolics into the saturated solvent (Kristensen et al., 2009; Macedo et al., 2021). The use of a pectinase pre-treatment before the 0.1% alkaline protease extraction was therefore regarded as unnecessary, thus saving time, energy, and resources to promote a more economically feasible extraction process.

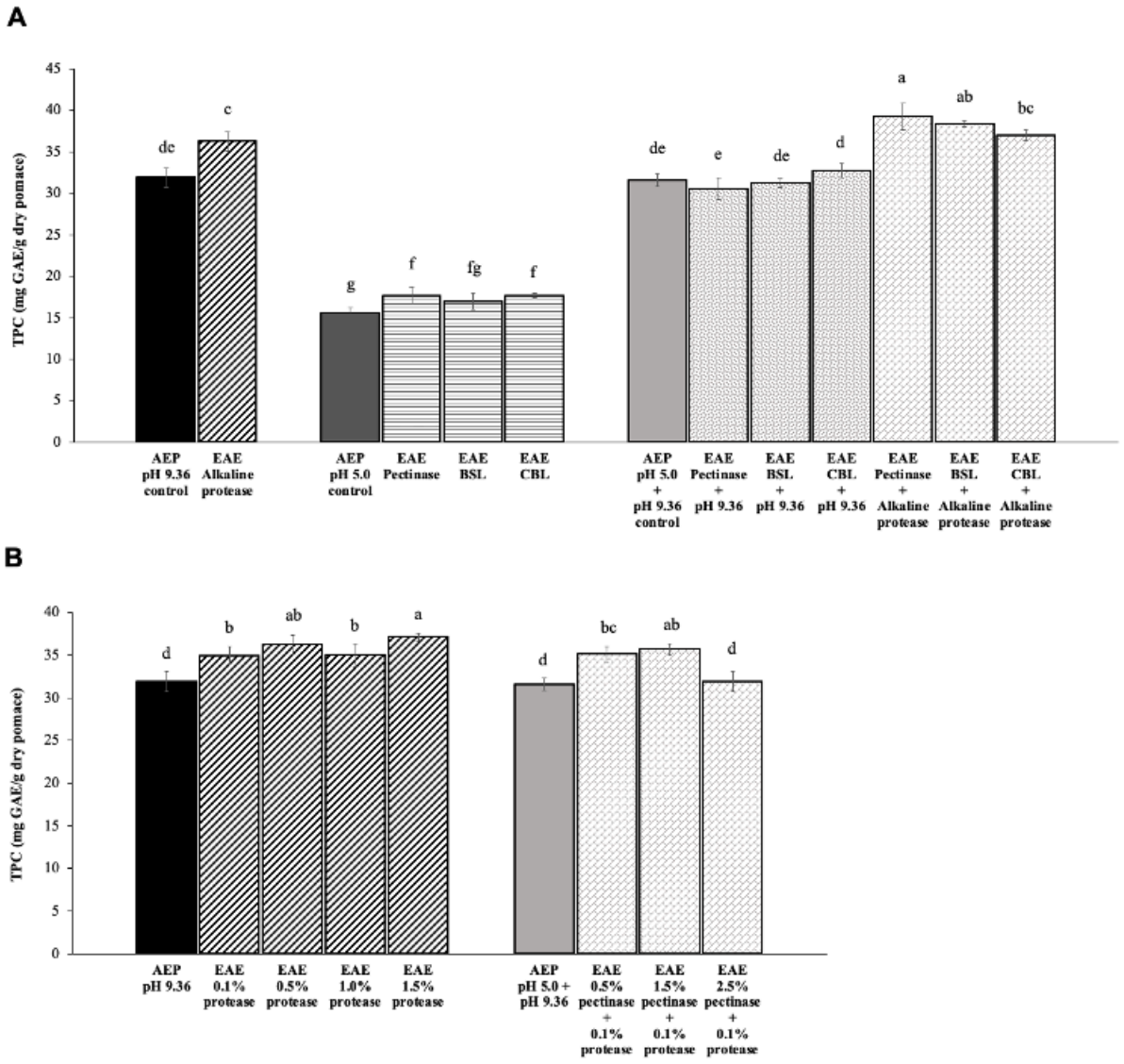


Figure 3.2. Effect of enzyme-assisted extraction (EAE) screening experiments on the phenolic content of unfermented Cabernet Sauvignon grape pomace extracts. [A] Screening of FoodPro® alkaline protease, pectinase, FoodPro® BSL, and FoodPro® CBL individually and in combination at 0.5% (w/w) loading. [B] Effects of FoodPro® alkaline protease and pectinase pre-treatment concentrations. All experiments were performed using a 1:50 SLR (g pomace/mL water) at 50 °C for 75 min with a 30 min pre-treatment when applicable. Different letters indicate significant ($p < 0.05$) differences in TPC amongst all treatments as indicated by Tukey's HSD test.

3.3.2. Effect of reaction time, SLR, and pH on the phenolic extractability of alkaline protease-assisted extraction

Upon selection of the 0.1% (w/w) FoodPro® alkaline protease extraction for all EAE treatments, the optimal extraction time, SLR, and slurry pH were identified by stepwise optimization process. Extraction temperature was not examined in these experiments because 50 °C has been shown to provide an adequate compromise between promoting solute diffusion and enzymatic hydrolysis while preventing degradation of thermolabile phenolics (Oreopoulou et al., 2019). The results of the kinetic study (Figure 3.3A) indicate that the total extraction time could be reduced from the initial 75 min (control, AEP) to 60 min while still maintaining high phenolic yields. Reduced extraction time when using alkaline protease to aid the extraction is likely the result of more rapid degradation of the grape cell components by catalyzing selective hydrolysis of structural proteins, thus aiding in a quicker release of phenolics (Gómez-García et al., 2012; Xu et al., 2014).

Next, the SLR of the extraction slurry was re-evaluated at 60 min to determine if water consumption could be reduced without negatively impacting TPC. Phenolic yields were reduced from 34.8 ± 0.99 mg GAE/g dry pomace (Figure 3.3B) to 31.6 ± 0.37 mg GAE/g dry pomace when SLR increased from 1:50 to 1:10 in the EAE. It is well understood that more dilute solutions will promote mass transfer of compounds due to the larger concentration gradient between the substrate and the solvent (Nayak et al., 2018; Pinelo et al., 2005). At the same time, larger volumes of solvent will require higher energy inputs during downstream processing to concentrate the bioactive compounds for further applications. Therefore, the 1:10 SLR, which generated a substantial TPC of 31.6 ± 0.37 mg GAE/g dry pomace at reduced water usage, was selected for continued optimization to explore a more cost-effective option for potential future commercialization efforts.

As previously shown in Chapter 2, increasing the slurry pH resulted in higher TPC in the extracts from unfermented Cabernet Sauvignon pomace. Since the FoodPro® alkaline protease maintains stable activity up to pH 10.5, a higher range of alkaline conditions was evaluated to elucidate the synergistic effects of pH and enzymatic activity on total phenolic yields. Compared to the aqueous control at pH 9.36, the addition of 0.1% (w/w) alkaline protease to the grape pomace slurry adjusted to pH 10.5 produced a two-fold increase in phenolic yield to a maximum TPC of 65.5 ± 3.24 mg GAE/g dry pomace (Figure 3.3C). Furthermore, the integration of both alkaline hydrolysis and protease-assisted extraction improved phenolic extraction by over 38% compared to the conventional hydroethanolic extraction using 60% ethanol (v/v). This phenomenon highlights the potential for EAE to serve as an alternative to conventional solvent extraction by releasing polyphenols, especially bound phenolics, from the grape cell matrix. While some enzyme-assisted extraction investigations have focused on the use of cellulases, tannases, and pectinases to hydrolyze residual sugars and structural carbohydrates for the release of phenolics (Chamorro et al., 2012; Martins et al., 2016), in this study, phenolic recovery was best induced using 0.1% (w/w) FoodPro® alkaline protease at pH 10.5 with a 1:10 SLR at 50 °C for 60 min. The SLR, temperature, and time parameters identified for EAE were used as the foundation for the MAE screening experiments.

While the use of alkaline extraction to recover phenolics from grape pomace is not yet widely reported, it is important to recognize that alkali is traditionally used as a pretreatment when cooking corn to produce masa, tortillas, and tortilla chips at commercial scales (de La Parra et al., 2007; Kasote et al., 2021). The nixtamalization process occurs at pH 9–13 using 0.5-1.5% (w/w) calcium hydroxide solution (also known as lime), which dissolves hemicellulose to improve digestibility, reduce antinutrients and mycotoxins, enhance calcium uptake, and increase

the bioaccessibility of phenolics. Additionally, the nixtamalization effluent can be recycled as a calcium- and phenolic-rich filtrate for the concentration of valuable compounds. Castro-Muñoz et al. (2016) reported that the retentate from ultrafiltration of nixtamalization wastewater contained 986 mg/L polyphenols and 3155 mg/L calcium at pH 13.28. This example emphasizes that alkaline conditions alone, even without the use of protease to hydrolyze cell wall structures, can still produce rich phenolic extracts. Further investigation of the production and mitigation of sodium and calcium salts during large scale extractions is required.

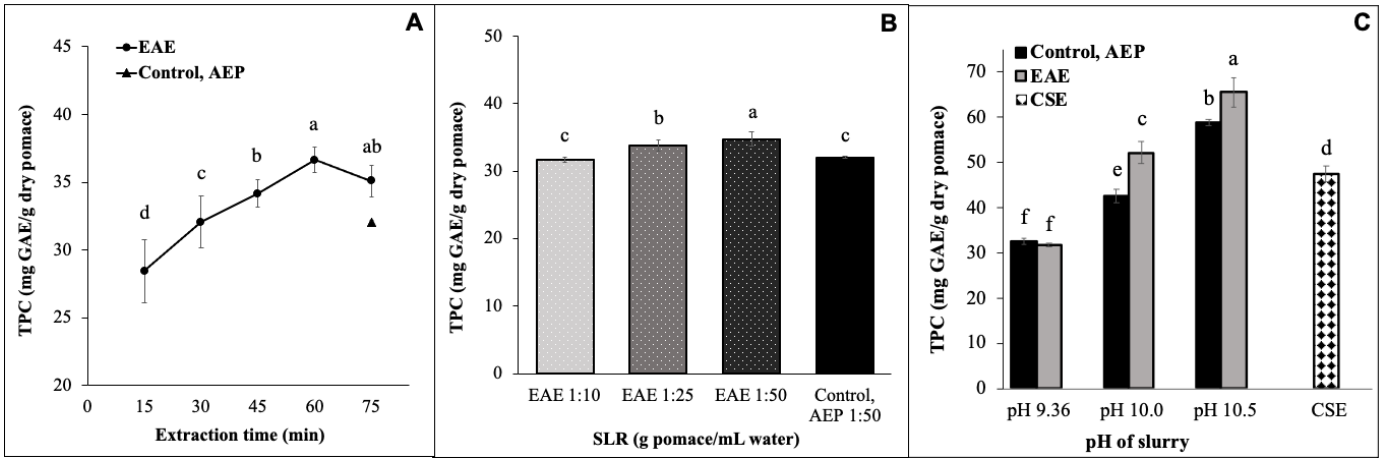


Figure 3.3. Selection of enzyme-assisted extraction (EAE) parameters: [A] Effect of extraction time using 0.1% protease (w/w), pH 9.36, 1:50 SLR, 50 °C (●) compared to the aqueous control at 75 min (▲); [B] Effect of solids-to-liquid ratio at pH 9.36, 50 °C, 60 min on TPC compared to the aqueous control; [C] Effect of increased pH on aqueous and enzyme-assisted extractions compared to conventional solvent extraction at 1:10 SLR, 50 °C, 60 min. Different letters indicate significant ($p < 0.05$) differences in TPC within each figure as indicated by Tukey's HSD test. AEP (control): aqueous extraction process. CSE: conventional solvent extraction (60% ethanol).

3.3.3. Effect of microwave-assisted extraction parameters on phenolic extraction

Microwave-assisted extraction parameters such as slurry pH, SLR, temperature, and time were first evaluated without the use of alkaline protease to better understand the unique impact of microwave radiation on releasing phenolics from the grape pomace matrix. Although similar extraction parameters were explored with EAE, they were re-examined with MAE due to the

different mechanisms of action induced by the different heating techniques. First, the grape pomace samples were adjusted to pH 9.36–12.0 at the start of the extraction to determine the best alkaline condition for MAE. Since the slurry pH could not be maintained while the sample was in the microwave, the final pH values ranged from 7.11– 11.6, respectively. Significant increases in TPC were observed with each increase in pH up to a maximum TPC of 79.7 ± 2.17 mg GAE/g dry pomace at pH 12.0 (Figure 3.4A). The positive linear trend with the use of alkaline conditions, also observed by Librán et al. (2013), is likely caused by the cleavage of ester- and ether-linked phenolics from proteins within the grape matrix.

Other reports of the use of MAE have shown positive effects on phenolic extraction from grape pomace (Álvarez et al., 2017; Brahim et al., 2014; Drosou et al., 2015; Li et al., 2011), but a direct comparison of data is challenging due to the lack of studies specifically employing alkaline conditions with unfermented Cabernet Sauvignon pomace samples. Drevelegka & Goula (2020) optimized MAE of phenolics from Agiorgitiko pomace using 42% ethanol at 408 W microwave power, 1:24 SLR, and 5 min, yielding a TPC of 36.44 ± 0.96 mg GAE/g dry pomace. Additionally, the use of alkaline extraction in MAE has shown improvements in phenolic yields from other plant materials, such as cocoa bean shell waste (Mellinas et al., 2020) (pH 12, 1:25 SLR, 97 °C, 5 min, 35.9 ± 0.9 mg GAE/g DW) and cranberry pomace (Davis et al., 2021), but alkaline methods have not yet been employed for Cabernet Sauvignon pomace during microwave processing.

Since the goal of this study was to integrate both MAE and FoodPro® alkaline protease, which begins to lose activity at pH values beyond 10.5, all further microwave-assisted screening experiments were adjusted to pH 11.5 to allow for a drop in pH to approximately pH 10.4 during

microwave extraction, thus encouraging maximum phenolic yields while maintaining enzymatic stability.

The SLRs of 1:10, 1:25, and 1:50 g pomace/mL water were evaluated at pH 11.5 and 50 °C for 60 min. Since no significant enhancement in phenolic extractability was observed when decreasing the SLR from 1:10 to 1:50, the 1:10 SLR was selected for subsequent experiments to reduce water usage (Figure 3.4B). The different trends observed with respect to the impact of SLR on phenolic extractability between MAE and EAE may be due to the different heating mechanisms and pH conditions of each treatment. In EAE, although the 1:10 SLR was selected to conserve water, the more dilute 1:50 SLR increased TPC in accordance with mass transfer principles. In MAE, however, phenolic extractability at higher SLR (i.e., 1:10 SLR) may have been enhanced by microwave radiation, which improved heating, cell wall disruption, and phenolic extraction despite the lower concentration gradient.

Increasing microwave temperature from 40 to 70 °C resulted in a maximum TPC (91.9 ± 0.94 mg GAE/g dry pomace) at 70 °C using pH 11.5, 1:10 SLR, and 60 min of extraction (Figure 3.4C). Interestingly, although the pH was initially adjusted to pH 11.5 prior to extraction, the final extract pH upon removal from the microwave was 7.8. This result suggests that elevated MAE temperatures may compensate for the decline in pH due to the release of phenolic acids into the solvent. Microwave heating causes localized pressure and temperature increases within the grape cells until the cells eventually burst, and this rupturing allows for the diffusion of intracellular solutes into the surrounding environment. Although there are concerns related to the degradation of thermolabile compounds, microwave-assisted extraction was found to maintain the stability of benzoic acids, benzoic aldehydes, cinnamic acids, catechins, coumarins, stilbenes, and flavonols at temperatures from 50 °C up to 100 °C for 20 min (Liazid et al., 2007). The selection of 70 °C

provides adequate thermal energy to drive mass transfer while also conserving resources to promote economic feasibility and environmental sustainability.

To evaluate MAE kinetics, samples were evaluated after 5, 15, 30, 45, and 60 min of extraction. Each condition used a 5 min ramp-up to achieve the set temperature of 70 °C with the remaining extraction time consisting of the time held at 70 °C. Figure 3.4D shows no significant difference in TPC at any time point, emphasizing the role of MAE in substantially reducing required extraction time to just 5 min of microwave exposure. After only 5 min in the microwave using pH 11.5 and 1:10 SLR at 70 °C, the TPC of the extract was 87.8 ± 0.38 mg GAE/g dry pomace. Compared to the 60-minute EAE control (pH 10.5, 1:10 SLR, 50 °C water bath), which resulted in a TPC of 58.8 ± 0.66 mg GAE/g dry pomace, the use of a 5-min MAE improved yields by nearly 50%. Constant extraction yields from 5 to 60 min suggests that, at the selected extraction parameters, phenolic extraction was exhaustive and further diffusion of the solute into the solvent could not occur. The rapid heat treatment in MAE is caused by the exposure of polar water molecules in the grape cells and surrounding solvent to the oscillating electromagnetic field. As water molecules begin to absorb microwave energy, their random motion generates friction and targeted heating within the sample (Chaturvedi, 2018). This mechanism encourages faster reaction times than conventional conductive heating.

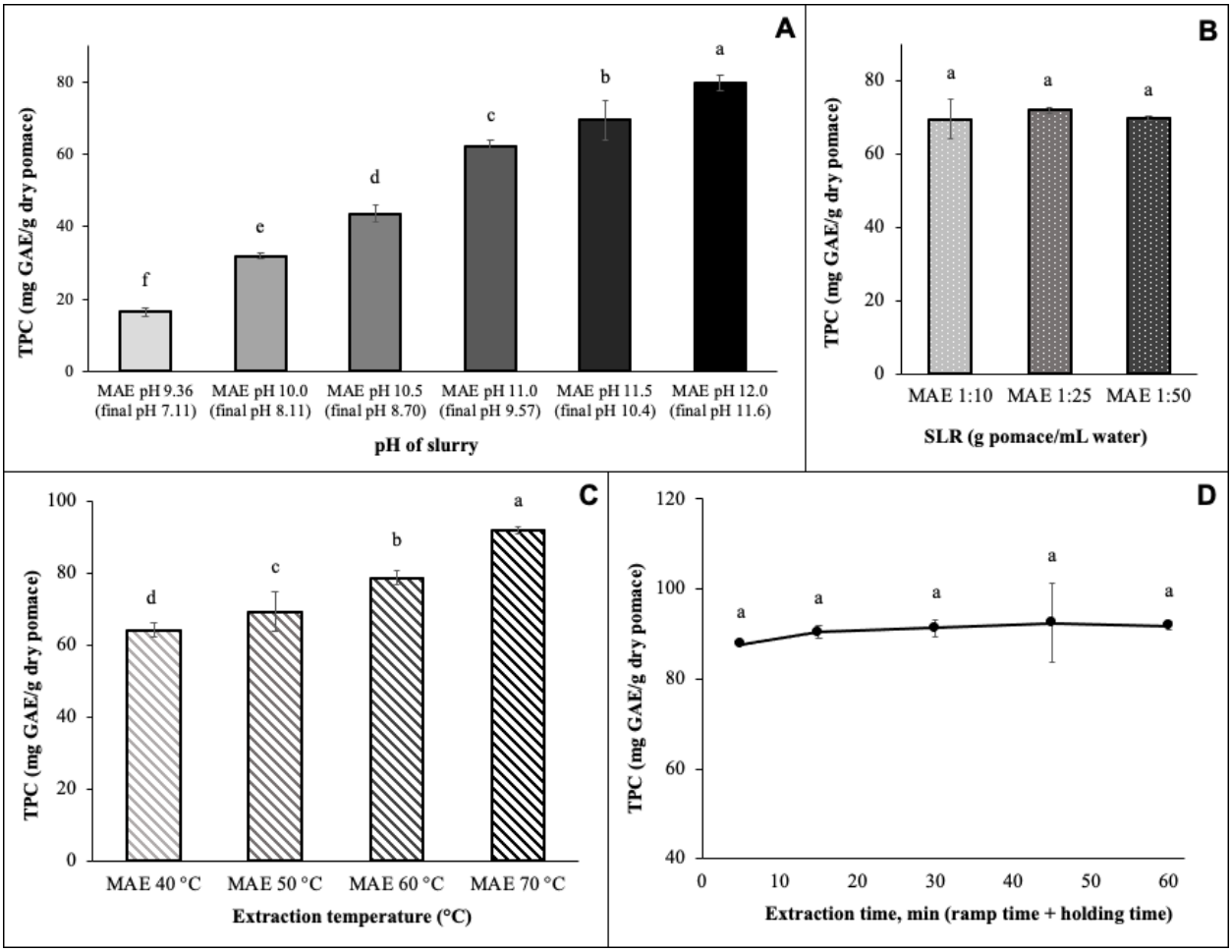


Figure 3.4. Effect of microwave-assisted extraction (MAE) parameters on TPC: [A] increasing alkaline pH at 1:10 SLR, 50°C, 60 min (5 min ramp + 55 min hold); [B] decreasing solids-to-liquid ratio at pH 11.5, 50 °C, 60 min (5 min ramp + 55 min hold); [C] increasing temperature at pH 11.5, 1:10 SLR, 60 min (5 min ramp + 55 min hold); and [D] increasing extraction time at pH 11.5, 1:10 SLR, 70 °C. Different letters indicate significant ($p < 0.05$) differences in TPC within each figure as indicated by Tukey's HSD test.

3.3.4. Integrated microwave enzyme-assisted extraction (MEAE)

Although 5 min was an adequate extraction time to achieve impressive phenolic yields using MAE, the extraction time was ultimately extended to 45 min to perform a preliminary evaluation of the role of enzyme application in the MEAE process. The hypothesis was that 5 min could not provide enough time to observe the full effects caused by both enzymatic and microwave activities, whereas 45 min could provide ample time to evaluate enzymatic extraction

kinetics while still minimizing total extraction time. The specific extraction time for MEAE was optimized in later screenings.

While 0.1% (w/w) protease was satisfactory for EAE, it was important to consider the potential need for a different enzyme dosage in MEAE due to the changes in heating mechanisms and vessel properties between the two techniques. Specifically, a higher enzyme dosage was explored from 0.1–2.5% (w/w) at pH 11.5, 1:10 SLR, and 70 °C for 45 min (5 min ramp + 40 min hold). As shown in Figure 3.5A, the use of 0.1– 2.0% (w/w) protease resulted in no change in the TPC of the MEAE extracts compared to the MAE control. In fact, the highest protease concentration of 2.5% (w/w) resulted in a slight decrease in TPC, which was similarly observed by Cheng et al. (2015) with a high loading (2.0–3.0%) of cellulase, papain, and pectinase for carbohydrate extraction. Although there was no increase in TPC using any of the protease concentrations during this MEAE screening trial, the minimum enzyme concentration of 0.1% (w/w) was selected for further optimization of MEAE to provide an opportunity to explore enzyme extraction kinetics while also being conscious of the associated costs.

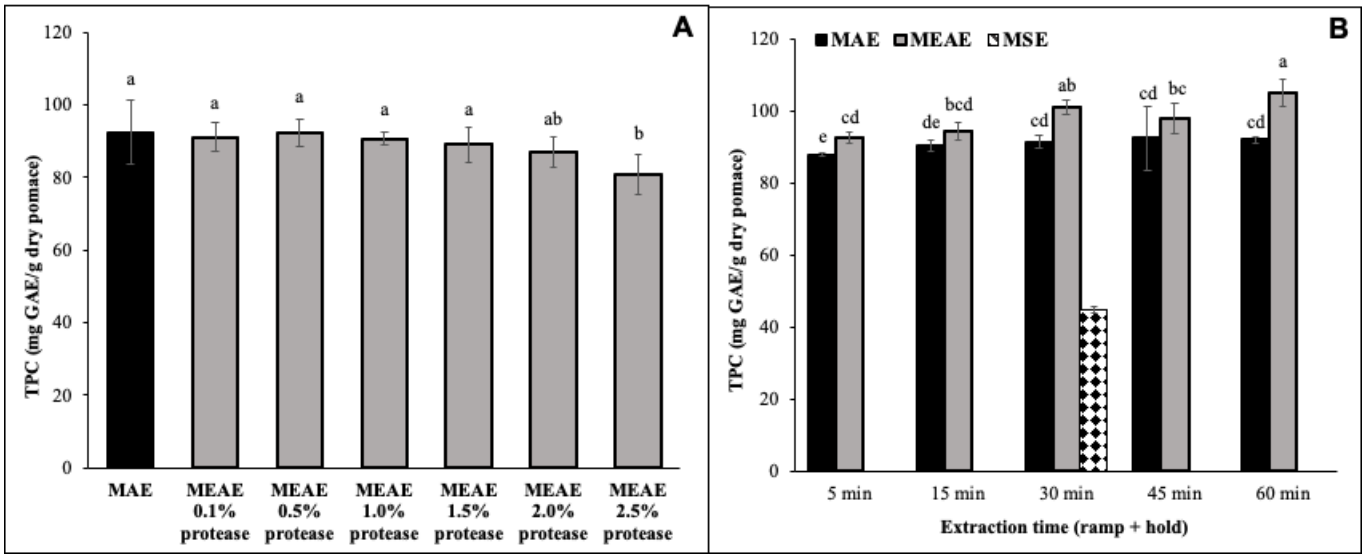


Figure 3.5. Effect of alkaline protease concentration (w/w) on TPC of microwave enzyme-assisted (MEAE) extracts at pH 11.5, 1:10 SLR, 70 °C, 45 min (5 min ramp + 40 min hold) [A]. Comparison of kinetic studies from MAE and MEAE experiments at pH 11.5, 1:10 SLR, 70 °C [B]. Different letters indicate significant differences ($p < 0.05$) in each figure as indicated by Tukey's HSD test. MAE: Microwave-assisted extraction. MSE: Microwave solvent extraction using 60% ethanol.

Because the initial reaction time selection of 45 min may have not been long enough to demonstrate the potential benefits of the protease in MEAE, a wider range of extraction times (5, 15, 30, 45, and 60 min) was evaluated for TPC using 0.1% (w/w) FoodPro® alkaline protease. Maximum TPC occurred after 60 min of extraction (105.1 ± 3.83 mg GAE/g dry pomace) but was not statistically different from the TPC at 30 min of extraction (100.9 ± 2.09 mg GAE/g dry pomace) (Figure 3.5B). Although the phenolic yield after just 5 min of MEAE exposure was impressive (92.4 ± 1.58 mg GAE/g dry pomace), a 30-min MEAE treatment provided higher TPC, so this sample was selected for further analysis of antioxidant properties and phenolic profiling. It is important to highlight that despite the small improvements in the TPC of the extracts produced by MEAE compared with MAE, a different phenolic profile could be expected for both extracts due to the role of the enzyme during the extraction. These results indicate that the combined effects

of microwave processing and enzyme-assisted extraction enabled a reduction in the total processing time and increased phenolic extractability compared to the results from the techniques when applied individually. The synergy of microwave enzyme-assisted extraction has also been studied by Görgüç et al. (2020) and Macedo et al. (2021) in the extraction of phenolics from sesame bran and olive pomace, respectively, but not thoroughly detailed for wine grape pomace. In one study, Jia et al. (2021) optimized the phenolic extraction (125.32 mg GAE/g dry weight) from Cabernet Sauvignon seeds using enzyme and microwave co-assisted salting-out extraction, but this method still relied on the use of 25% (w/w) ethanol and explored the activity of pectinase under acidic conditions (pectinase amount of 540 U/g at pH 4.5). Additionally, Jia et al. (2021) only focused on extracting phenolics from grape seeds, while the present study also included Cabernet Sauvignon grape skins to explore the additional valuable polyphenolic components that this fraction contributes. The results of the present study, therefore, provide a new approach to MEAE at alkaline conditions using the unfermented Cabernet Sauvignon grape pomace feedstock.

Since the highest TPC across all experiments was produced using MEAE at 70 °C with a 1:10 SLR for 30 min, this extraction condition was also performed with 60% ethanol to provide a comparison to conventional solvent techniques. As shown in Figure 3.5B, microwave solvent extraction (MSE) was less effective than all MAE and MEAE treatments. In fact, the use of 60% ethanol in the microwave generated a TPC over two-times lower than the TPC generated using water at the same microwave extraction conditions. The low phenolic yields from MSE could be explained by the lower polarity and dielectric constant of ethanol compared to water. The use of 60% ethanol slightly decreased the polarity of the solvent, which reduced the extraction of polar phenolic compounds since microwave radiation is better absorbed by highly polar solvents. These

results reinforce the opportunity for substituting ethanol with pure water under optimized microwave conditions for the recovery of bioactive plant compounds.

3.3.5. Antioxidant activities of grape pomace extracts

The antioxidant capacities of the extracts resulting from the optimal EAE, MAE, MEAE, and control samples were determined using ABTS and ORAC assays. Both analyses were performed to provide a more holistic perspective of the *in vitro* biological properties of grape pomace extracts. The use of multiple antioxidant capacity tests is commonly encouraged due to the different radical scavenging mechanisms involved in each test: ABTS measures antioxidant activity by the single-electron transfer (SET) method while ORAC uses the hydrogen atom transfer (HAT) reaction to measure peroxyl radical decomposition (Huang et al., 2005; Speisky et al., 2017).

Overall, similar trends were observed between the radical scavenging activities from ABTS and ORAC assays across all treatments. Notably, there was no significant difference between the antioxidant power obtained from ABTS or ORAC assays for CSE, MSE, MAE, and MEAE extracts (Table 3.1). However, AEP and EAE extracts showed slightly lower chemical antioxidant activities despite their relatively high phenolic yields, which highlights the importance of understanding the phenolic composition of the extracts. The use of 60% ethanol likely improved the antioxidant activity in CSE and MSE extracts due to a shift in their associated phenolic profiles. The results of the untargeted metabolomics profiling (Supplementary Materials—Table S3.1) show that the hydroethanolic extracts were richer in the anthocyanin malvidin-3,5-diglucoside, which has been associated with driving antioxidant activity in grape extracts (Jiménez-Moreno et al., 2019; Rivero-Pérez et al., 2008). The higher relative content of malvidin-3,5-diglucoside in the hydroethanolic extracts is likely due to the stability of anthocyanins in acidic

environments, where the flavylium ion of anthocyanins is in its dominant protonated form (Seeram et al., 2001). Therefore, it is likely that the solvent specificity of some phenolics in CSE and MSE extracts may have improved their antioxidant activity in 60% ethanol. It is also possible that the slurry exposure to air and light during AEP and EAE, which were performed in open vessels compared to sealed vessels for MSE, MAE, and MEAE extracts within the microwave, might have reduced the antioxidant power of the grape pomace phenolics due to oxidation (Lafka et al., 2007)

Despite this phenomenon, it is evident that the use of microwave-assisted extraction in the presence or absence of enzymes maintained the bioactivity of the grape pomace extracts to values similar to those of CSE (not statistically different at $p < 0.05$) while using the alkaline slurry as the solvent at moderate extraction conditions. Kumar et al. (2020) also observed the cumulative effects of integrating MAE and EAE on improving the antioxidant power (FRAP) of pomegranate peel extracts compared to the use of either method or CSE alone. The promising bioactivity of both MAE and MEAE extracts, which can be produced at much shorter reaction times compared with AEP and EAP and without the use of flammable solvents in MSE and CSE, supports the use of this strategy in developing functional ingredients for food, beverage, cosmetic, or pharmaceutical applications. Further research should evaluate the efficacy of the grape pomace extracts in cell culture studies and *in vivo* trials to determine the bioavailability and bioaccessibility of the phenolics for biological radical scavenging.

Table 3.1. Total phenolic content and antioxidant activities of grape pomace extracts from optimal conditions for each treatment

Treatment	Extraction conditions	TPC mg GAE/g dry pomace	ABTS µmol TE/g dry pomace	ORAC µmol TE/g dry pomace
CSE	60% ethanol, 1:10 SLR, 60 min, 50 °C	47.3 ± 1.78^{e}	298 ± 0.99^{a}	788 ± 34.8^{a}
AEP	pH 10.5, 1:10 SLR, 60 min, 50 °C	58.8 ± 0.66^{d}	211 ± 17.7^{c}	396 ± 19.5^{c}
EAE	0.1% protease, pH 10.5, 1:10 SLR, 60 min, 50 °C	65.5 ± 3.24^{c}	261 ± 4.86^{b}	522 ± 43.6^{b}
MSE	60% ethanol, 1:10 SLR, 30 min, 70 °C	44.6 ± 1.47^{e}	293 ± 1.85^{a}	747 ± 97.0^{a}
MAE	pH 11.5, 1:10 SLR, 30 min, 70 °C	91.3 ± 1.79^{b}	297 ± 0.66^{a}	710 ± 44.7^{a}
MEAE	0.1% protease, pH 11.5, 1:10 SLR, 30 min, 70 °C	100.9 ± 2.09^{a}	297 ± 0.59^{a}	793 ± 88.5^{a}

Different lowercase letters in the same column indicate significant differences ($p < 0.05$) within that column. Values represent mean ± SD ($n = 9$).

3.3.6. Phenolic profiling by untargeted metabolomics approach

The phenolic compositions of the six selected grape pomace extracts shown in Table 3.1 were also investigated by an untargeted metabolomics approach to better identify the pool of secondary metabolites associated with unfermented Cabernet Sauvignon grape pomace. This strategy aimed to highlight similarities and differences among the phenolic compounds identified across the conventional and alternative extraction methods. Overall, 37 phenolic compounds were identified from the 263 total annotated grape compounds, which were part of the 1,950 total identified metabolites including sugars, amino acids, and lipids. The average (n = 3) peak heights reported for each sample (Supplementary Materials—Table S3.1) were determined by dividing each metabolite peak by the sum of all peak heights for all identified metabolites for each sample. The reported relative content of each identified and annotated phenolic compound was then normalized as a fraction (%) of the total peak heights from all phenolics in the sample. It is important to note that the sum of the total phenolic peak heights varied greatly across extraction treatments with the ethanol extractions yielding total peak heights that were ten-times greater than the aqueous extractions.

The phenolic subclass with the greatest constituents across all extracts was flavonoid glycosides. The phenolic compositions from each treatment are illustrated in Figure 3.6 with

similar compositional trends between CSE and MSE, AEP and EAE, and MAE and MEAE. The extracts of CSE and MSE were rich in flavanols, anthocyanins (i.e, malvidin 3,5-diglucoside), and flavonols. The alternative green extraction methods (AEP, EAE, MAE, and MEAE) contained a higher proportion of hydroxycinnamic and hydroxybenzoic acids compared to the hydroethanolic extractions, and the MAE and MEAE methods also showed increases in the relative content of the stilbene trans-piceid.

Malvidin 3,5-diglucoside and procyanidin B2 isomers were the most abundant compounds identified in the extracts from CSE and MSE (Supplementary Materials—Table S3.1). This finding suggests that the hydroethanolic solvent promoted dissolution of these flavanols and anthocyanins. Other phenolic identifications that were unique to the hydroethanolic extractions include kaempferol and kaempferol 3-alpha-L-arabinopyranoside (flavonols), tricin and jaceoside (flavones), and iridin (isoflavone). Tricin, jaceoside, and iridin have not been widely reported in the literature as being associated with Cabernet Sauvignon skins and seeds.

The AEP and EAE extracts contained a higher relative content of the following compounds, which in total represented over 85% of the total peak height for each treatment (Supplementary Materials—Table S3.1): trans-melilotoside isomers (hydroxycinnamic acids), gentisic acid-5-O-glucoside (hydroxybenzoic acid), and isorhamnetin 3-galactoside and myricitrin (flavonols). The AEP and EAE extracts contained a lower proportion of flavanols (e.g., catechin, epicatechin, and procyanidins) and anthocyanins (e.g., malvidin 3,5-diglucoside) compared to the relative contents of these compounds in the other extraction treatments. Since these compounds are strongly associated with providing antioxidant activity (Iacopini et al., 2008; Lachman et al., 2009; Yilmaz & Toledo, 2003), this likely explains the lower ABTS and ORAC antioxidant activities of AEP and EAE extracts as shown in Table 3.1.

Compared to AEP and EAE, the composition of the MAE and MEAE extracts shifted towards flavanols, specifically procyanidin B2 isomers (Supplementary Materials—Table S3.1), which relates these extracts to those obtained using the hydroethanolic solvents. In addition to flavanols, MAE and MEAE extracts also contained high relative contents of trans-melilotoside and gentisic acid-5-O-glucoside. Compared to the MSE samples, MAE and MEAE techniques also increased the relative contents of p-coumaric acid, epicatechin monogallate, catechins, and trans-piceid. Trans-piceid is a resveratrol glucoside considered to be the most abundant form of resveratrol in nature with physiological importance in inhibiting platelet aggregation (Romero-Pérez et al., 1999).

The phenolic profiles in Figure 3.6 highlight the capability of untargeted approaches to illuminate a wider variety of phenolics that may be otherwise overlooked through targeted methods. For example, an abundance of glycosidic forms of phenolic acids and flavonoids were identified in the grape pomace extracts that may not be typically included as standards in targeted methods. The pervasiveness of these flavonoid glycosides throughout the grape pomace extracts may be explained by the steric hindrance created by sugar moieties and the protection that this acylation may offer against polyphenolic degradation at alkaline conditions (Fleschhut et al., 2006). This result also emphasizes the role of the starting material composition, which was high in residual sugars due to the pomace being collected prior to fermentation. Notably, this study highlights the effect that upstream winemaking methods can have on the downstream chemical and biological properties of the grape pomace extracts. Further, polyphenols like naringenin-7-O-glucoside, salviaflaside, and secoisolariciresinol that are commonly associated with citrus, self-heal herb (Prunella vulgaris), and flaxseed, respectively, were identified in the grape extracts (Supplementary Materials—Table S3.1). These findings elucidate potential relationships between

the phytochemistry of various plant species and offer insight into shared metabolic pathways and mechanisms of action of these bioactives. However, since the data shown in Figure 3.6 was normalized based on the sum of all phenolic peak heights within the sample, further quantitation is required to understand how extraction treatments can affect the resulting phenolic contents of the extracts.

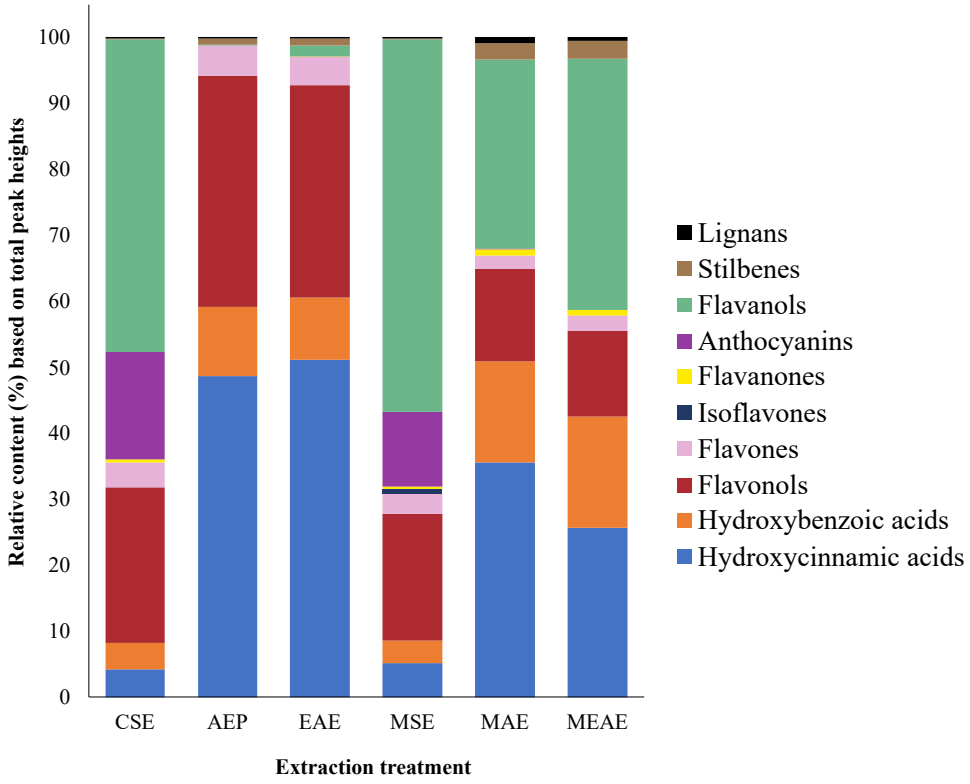


Figure 3.6. Phenolic compositions of the grape pomace extracts identified using an untargeted metabolomics approach.

3.3.7. Effects of extraction treatments on the microstructure of grape cell matrix

Images of the grape pomace insoluble fractions were captured using scanning electron microscopy (SEM) to illustrate the effect of the various extraction treatments on the microstructure of the grape cell matrix. As shown in Figure 3.7B-G, different combinations of ethanol, alkali, protease, and microwave radiation degraded the grape cell wall and altered the

surface morphology of the grape pomace samples compared to the untreated control (Figure 3.7A).

The freshly ground grape pomace in the untreated control sample shows a smooth, tightly packed surface (Figure 3.7A). Although this material was exposed to a short mechanical pre-treatment using mortar and pestle to reduce the sample particle size to a finer paste, the surface structure of the particles remained relatively continuous and intact. The use of 60% ethanol in CSE (Figure 3.7B) did not cause significant morphological differences to the cell structure as compared to the untreated control (Figure 3.7A), which reflects the low phenolic extraction trend shown in Table 3.1. It is possible that during the alkaline extractions at pH 10.5 (Figure 3.7C-D), fragmentation and disintegration of the structural proteins and fibrous carbohydrates revealed a honeycomb appearance with a highly porous matrix. The addition of alkaline protease (Figure 3.7D) in EAE shows the enhanced permeability of the cell matrix caused by enzymatic hydrolysis and the large pores produced by the diffusion of compounds across the grape cell wall. These results are also in agreement with the improvement in phenolic extractability of EAE compared to AEP (Table 3.1).

More pronounced rupturing effects were evident with the use of microwave-assisted extraction (Figure 3.7E-G). The combination of higher temperatures and pressure within the grape cell matrix using microwave heating resulted in the bursting of the cell structure. This cellular damage is reflective of the high phenolic extraction yields recovered by MAE and MEAE. In addition, disruption of the grape cell matrix via microwave radiation provided more surface area and better accessibility of the material to enzymatic attack, thus improving overall phenolic extraction with the addition of FoodPro® alkaline protease in MEAE (Figure 3.7G). The SEM image of the MEAE process illustrates the severe loss in the structural integrity of the

grape pomace and shows the mechanism for liberating bound phenolics from the sample. Similar effects using combined alkaline and microwave extraction systems were observed by Görgüç et al. (2020) in the extraction of proteins and phenolics from sesame bran.

When comparing the use of 60% ethanol as the extraction solvent, the MSE sample (Figure 3.7E) shows more intense fragmentation of the grape cell matrix compared to the CSE sample (Figure 3.7B). This may be caused by a more intense heating process in the microwave at 70 °C and the higher dissipation factor of ethanol (Mandal et al., 2006). While ethanol absorbs microwave energy less efficiently than water due to its lower dielectric constant, it disperses the heat to surrounding molecules much faster, which resulted in severe cellular damage to the MSE sample and may have degraded some heat-sensitive phenolics, as suggested by its low phenolic content (Table 3.1). Xue et al. (2018) also observed intensified structural changes in blueberry powder using 60% ethanol as the solvent with increasing microwave power (100–400 W). Similarly, Özbek et al. (2019) evaluated the use of hydroethanolic solvents with and without microwave irradiation to extract phenolics from pistachio hulls, and a more extensive cellular rupture was reported with the aid of a 4.5-min, 140 W microwave treatment. The MSE method also proved to be less effective in phenolic extraction than the other microwave extractions (MAE and MEAE), which emphasizes the role of alkaline conditions in releasing and solubilizing phenolics from cell wall structural components. Overall, SEM provided visual representations of the morphological changes to the grape pomace microstructures across the various extraction treatments. The mechanism of phenolic liberation was best exemplified using MEAE, where the effects of cellular rupture by microwave energy were also supported by the structural damage caused by proteolytic activity.

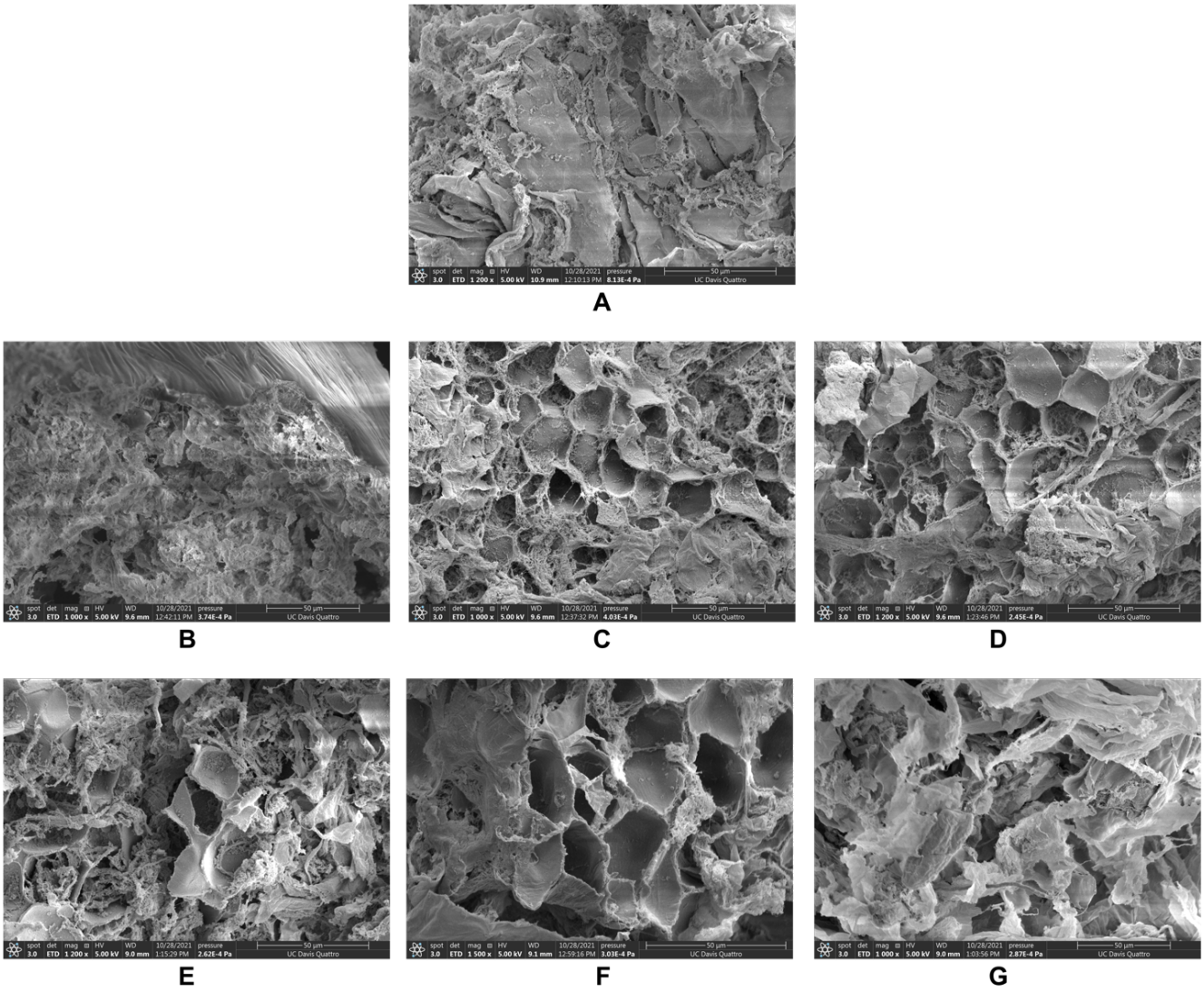


Figure 3.7. SEM images of grape pomace insoluble fractions obtained from various treatments at optimal conditions: [A] untreated control at 1200x, [B] CSE at 1000x, [C] AEP at 1000x, [D] EAEP at 1200x, [E] MSE at 1200x, [F] MAE at 1500x, and [G] MEAE at 1000x.

3.4. Conclusion

Wine grape pomace is an underutilized waste stream of the winemaking process that offers an abundant source of bioactive components, especially phenolics, for food and health applications. Green extraction technologies using water as the only solvent were successfully applied as promising, eco-friendly alternatives to conventional ethanol extraction. In this study,

an integrated design including alkaline conditions, protease-assisted extraction, and microwave radiation was developed to extract phenolics from Cabernet Sauvignon pomace collected prior to fermentation as a byproduct of a special white winemaking process. Microwave processing, in the presence or absence of enzymes, was an effective and rapid strategy to disrupt the grape pomace cell wall using water as the only solvent, which was reflected by higher phenolic extraction at reduced extraction times and intensified cellular damage as illustrated by SEM imaging. While AEP and EAE achieved phenolic contents of 58.8 ± 0.66 and 65.5 ± 3.24 mg GAE/g dry pomace, respectively, after 60 min of extraction at 50°C, MAE and MEAE increased phenolic extractability to 91.3 ± 1.79 and 100.9 ± 2.09 mg GAE/g dry pomace, respectively, after 30 min at 70 °C, yielding over two times the TPC of the ethanol extracts.

Importantly, while the *in vitro* antioxidant activities (by ABTS and ORAC methods) of AEP and EAP extracts were lower than the ones from CSE, which is considered the standard technique for phenolic extraction, MAE and MEAE extracts had similar activities to the CSE extract. These results highlight the impact of the different extraction methods on the selective extraction of phenolic compounds, which was elucidated using an untargeted metabolomics approach. The combined use of alkaline water, enzyme, and microwave processing shifted the phenolic profile of the MEAE extracts towards a higher proportion of hydroxycinnamic acids, hydroxybenzoic acids, stilbenes, and lignans but a lower proportion of flavanols, anthocyanins, and flavonols compared to the ethanol extracts. Future evaluation of *in vivo* antioxidant tests and delivery mechanisms for improving the bioaccessibility and bioavailability of these compounds for large-scale food, cosmetic, and pharmaceutical industries is warranted.

3.5. Acknowledgements

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3.6. Supplementary Materials

Table S3.1. Average peak heights and relative contents of phenolic compounds in grape pomace extracts as identified by untargeted metabolomics

Polyphenol class	Compound	CSE				AEP				EAE				MSE				MAE				MEAE			
		Avg. peak height*	SD	CV (%)	Rel. content (%)	Avg. peak height*	SD	CV (%)	Rel. content (%)	Avg. peak height*	SD	CV (%)	Rel. content (%)	Avg. peak height*	SD	CV (%)	Rel. content (%)	Avg. peak height*	SD	CV (%)	Rel. content (%)	Avg. peak height*	SD	CV (%)	Rel. content (%)
Phenolic acids																									
Hydroxycinnamic acids	*p*-Coumaric acid					129856	18089	14	**1.06**	146400	8856	6	**1.23**	13923	2855	21	**0.02**	489263	57154	12	**5.23**	439842	54489	12	**6.57**
	Salviaflaside	313652	42693	14	**0.41**									195834	4900	3	**0.29**								
	trans-Melilotoside Isomer A	2020144	121652	6	**2.61**	2053339	323501	16	**16.75**	1861943	145499	8	**15.68**	1432143	276519	19	**2.14**	1480399	195233	13	**15.83**	1185451	226405	19	**17.71**
	trans-Melilotoside Isomer B	862045	109817	13	**1.11**	3550268	521834	15	**28.96**	3815586	291077	8	**32.14**	1701465	250119	15	**2.55**	1230587	242892	20	**13.15**				
	trans-Melilotoside Isomer C	39089	8513	22	**0.05**	229013	33468	15	**1.87**	246798	25242	10	**2.08**	110279	19358	18	**0.17**	128422	11970	9	**1.37**	94827	22005	23	**1.42**
Hydroxybenzoic acids	Gentisic acid-5-*O*-glucoside	2566548	264641	10	**3.32**	1280779	159188	12	**10.45**	1116194	68318	6	**9.40**	1838023	275902	15	**2.75**	1434404	90905	6	**15.33**	1118785	142623	13	**16.71**
	Vanillic acid glucoside	566054	78944	14	**0.73**									450381	47127	10	**0.67**					4805	230	5	**0.07**
Flavanoids																									
Flavonols	Kaempferol	109206	23475	21	**0.14**									113058	13164	12	**0.17**								
	Kaempferol 3-alpha-L-arabinopyranoside	197766	36937	19	**0.26**									237351	36528	15	**0.36**								
	Quercetin	1078919	27159	3	**1.40**																				
	Quercitrin	423476	62065	15	**0.55**	56305	5976	11	**0.46**	40176	3970	10	**0.34**	324787	27799	9	**0.49**	18297	3228	18	**0.20**				
	Quercetin 3-*O*-glucuronide	2244399	424604	19	**2.90**	433704	71211	16	**3.54**	393689	24901	6	**3.32**	1616234	229080	14	**2.42**	234427	39093	17	**2.51**				
	Quercetin 3,4'-diglucoside	348890	41110	12	**0.45**	99370	20496	21	**0.81**	85381	5854	7	**0.72**	234966	10175	4	**0.35**	27863	3924	14	**0.30**	20075	3223	16	**0.30**
	Isorhamnetin 3-galactoside	5703567	217315	4	**7.37**	1862646	222948	12	**15.20**	1640648	64148	4	**13.82**	4309919	604753	14	**6.45**	419883	75294	18	**4.49**	325993	71716	22	**4.87**
	Isoquercitin	1154733	259241	22	**1.49**	72725	9038	12	**0.59**	66477	8458	13	**0.56**	860044	97722	11	**1.29**								
	Myricetin	56853	6397	11	**0.07**					1288	61	5	**0.01**									1600	470		**0.02**
	Myricitrin	6171672	658602	11	**7.98**	1604208	236388	15	**13.09**	1422443	105739	7	**11.98**	4493196	430736	10	**6.73**	545422	94355	17	**5.83**	470606	112033	24	**7.03**
	Rutoside (rutin)	159446	13767	9	**0.21**	33516	1509	5	**0.27**	28752	4938	17	**0.24**	122480	26696	22	**0.18**	29171	1862	6	**0.31**	31743	4374	14	**0.47**
	Syringetin 3-glucoside	550136	155828	28	**0.71**	142712	7488	5	**1.16**	147256	17761	12	**1.24**	488099	95249	20	**0.73**	33509	5990	18	**0.36**	25213	5599	22	**0.38**
Flavones	Luteolin-7-glucoside	2081955	177915	9	**2.69**	561618	87839	16	**4.58**	508975	50967	10	**4.29**	1435897	158105	11	**2.15**	187957	31865	17	**2.01**	152349	38975	26	**2.28**
	Tricin	740380	146731	20	**0.96**									509152	35479	7	**0.76**								
	Jaceoside	155620	45011	29	**0.20**									113350	20022	18	**0.17**								
Isoflavones	Iridin													482282	79462	16	**0.72**								
Flavanones	Naringenin-7-*O*-glucoside	150334	10783	7	**0.19**									122113	19518	16	**0.18**	64930	14089	22	**0.69**	43455	6771	16	**0.65**
	Astilbin Isomer A	144514	24311	17	**0.19**					8106	644	8	**0.07**	117322	12556	11	**0.18**	27032	2883	11	**0.29**	17288	2392	14	**0.26**
Anthocyanins	Malvidin 3,5-diglucoside	12622735	1631047	13	**16.32**									7585091	1343802	18	**11.36**	3053	672	22	**0.03**				
Flavanols	(-)-Epichatechin	87005	9391	11	**0.11**									204862	30942	15	**0.31**	24274	6527	27	**0.26**				
	Epicatechin monogallate	346604	32032	9	**0.45**					3998	441	11	**0.03**	630848	51239	8	**0.94**	302417	24707	8	**3.23**	269514	43955	16	**4.03**
	Catechin Isomer A	1508554	160400	11	**1.95**									2792666	371052	13	**4.18**								
	Catechin Isomer B													4021027	601359	15	**6.02**	16646	1746	10	**0.18**	9010	1790	20	**0.13**
	Catechin Isomer C																	377072	59277	16	**4.03**	385528	112373	29	**5.76**
	Procyanidin B2 Isomer A													2632163	442677	17	**3.94**	111025	8281	7	**1.19**				
	Procyanidin B2 Isomer B	12274020	1017989	8	**15.87**					192973	44556	23	**1.63**	10323849	1267598	12	**15.46**	1677220	235471	14	**17.93**	1702788	95255	6	**25.43**
	Procyanidin B2 Isomer C	15708053	1465909	9	**20.31**	2630	340	13	**0.02**					12232329	1948088	16	**18.31**	179822	30078	17	**1.92**	174864	45144	26	**2.61**
	Procyanidin C1	6736328	1013614	15	**8.71**									4875965	651683	13	**7.30**								
Stilbenes	*trans*-Piceid	100465	12289	12	**0.13**	116024	22243	19	**0.95**	116369	12275	11	**0.98**	82841	13724	17	**0.12**	231590	17705	8	**2.48**	182812	26911	15	**2.73**
Lignans	Secoisolariciresinol	115459	5601	5	**0.15**	28994	4040	14	**0.24**	28578	6293	22	**0.24**	86119	16900	20	**0.13**	79878	6674	8	**0.85**	38456	6584	17	**0.57**
Sum of peak heights:		**77338619**				**12257706**				**11872031**				**66790058**				**9354564**				**6695005**			

*Average peak heights are the result of triplicate samples

Table S3.2. Method parameters for untargeted phenolic profiling by Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap™ MS

Polyphenol class	Compound	Species	*m/z*	ESI mode	RT
Phenolic acids					
Hydroxycinnamic acids	*p*-Coumaric acid	[M+H]+	165.0546	positive	1.382
	Salviaflaside	[M-H]-	521.1303	negative	2.157
	Melilotoside Isomer A	[M-H]-	325.0930	negative	0.526
	Melilotoside Isomer B	[M-H]-	325.0931	negative	0.695
	Melilotoside Isomer C	[M-H]-	325.0931	negative	1.184
Hydroxybenzoic acids	Gentisic acid 5-*O*-glucoside	[M-H]-	315.0724	negative	0.489
	Vanillic acid glucoside	[M-H]-	329.0880	negative	1.297
Flavanoids					
Flavonols	Kaempferol	[M+H]+	287.0551	positive	4.431
	Kaempferol 3-alpha-L-arabinopyranoside	[M+Na]+	441.0818	positive	2.535
	Quercetin	[M-H]-	301.0356	negative	3.731
	Quercitrin	[M+H]+	449.1079	positive	2.465
	Quercetin 3-*O*-glucuronide	[M+H]+	479.0823	positive	2.079
	Quercetin 3,4'-diglucoside	[M-H]-	625.1416	negative	1.503
	Isorhamnetin 3-galactoside	[M-H]-	477.1040	negative	2.563
	Isoquercitin	[M+H]+	465.1031	positive	2.111
	Myricetin	[M-H]-	317.0306	negative	2.893
	Myricitrin	[M+Cl]-_[M-H]-	499.0652_463.0886	negative	2.051
	Rutoside (rutin)	[M-H]-	609.1471	negative	1.950
	Syringetin 3-glucoside	[M+Na]+	531.1110	positive	2.671
Flavones	Luteolin-7-glucoside	[M-H]-	447.0936	negative	2.415
	Tricin	[M+H]+	331.0806	positive	1.897
	Jaceoside	[M+H]+	331.0837	positive	3.856
Isoflavones	Iridin	[M+FA-H]-	567.1361	negative	2.521
Flavanones	Naringenin-7-*O*-glucoside	[M-H]-	433.1144	negative	2.683
	Astilbin Isomer A	[M-H]-	449.1093	negative	2.069
Anthocyanins	Malvidin 3,5-diglucoside	[Cat-2H-C6H8O4]-	509.1302	negative	1.572
Flavanols	(-)-Epichatechin	[M+Cl]-	325.0484	negative	1.224
	Epicatechin monogallate	[M-H]-	441.0831	negative	1.912
	Catechin Isomer A	[M+CHO2]-	335.0774	negative	0.969
	Catechin Isomer B	[M+H]+	291.0863	positive	1.044
	Catechin Isomer C	[M+H]+	291.0864	positive	0.555
	Procyanidin B2 Isomer A	[M+H]+	579.1498	positive	0.494
	Procyanidin B2 Isomer B	[M-H]-	577.1353	negative	0.468
	Procyanidin B2 Isomer C	[M-H]-	577.1354	negative	0.626
	Procyanidin C1	[M-H]-	865.1991	negative	1.058
Stilbenes	*trans*-Piceid	[M-H]-	389.1245	negative	1.982
Lignans	Secoisolariciresinol	[M+FA-H]-	407.1713	negative	2.661

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