OPTIMIZING PROANTHOCYANIDIN EXTRACTION FROM GRAPE SEEDS IN WINERY WASTES

By

GOVINDA SAPKOTA

Bachelor of Science in Agriculture

Tribhuvan University

Kathmandu, Nepal

2013

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 2018

OPTIMIZING PROANTHOCYANIDIN EXTRACTION FROM GRAPE SEEDS IN WINERY WASTES

Thesis Approved:

Niels Maness

Thesis Adviser

Bruce Dunn

William McGlynn

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Dr. Niels Maness for his invaluable guidelines, assistance and motivation. I find myself lucky to get to work as a graduate student with Dr. Maness, who provided me with such a wonderful opportunity to be a part of this research project. Secondly, I express my acknowledgements to Dr. Bruce Dunn and Dr. William McGlynn for their help and support to complete my research. I am also thankful to Dr. Mark Payton for his help in data analysis. I would also like to thank Ms Donna Chrtz for her help and support to complete this research.

I would like to convey my gratefulness to my family for their never-ending support and blessings which have always made me achieve my dreams. I would also like to thank all my friends for their support and encouragement.

Acknowledgements reflect the views of the author and are not endorsed by committee members or Oklahoma State University

Name: GOVINDA SAPKOTA

Date of Degree: DECEMBER, 2017

Title of Study: OPTIMIZING PROANTHOCYANIDIN EXTRACTION FROM GRAPE SEEDS IN WINERY WASTES

Major Field: HORTICULTURE

Abstract: Grape seeds have been reported to be a good source of phenolic compounds accounting for 5% to 8% of total seed weight on a dry basis. There is not an established optimum procedure for phenolics extraction. The main objective of this study was to optimize extraction conditions, and then develop a partial extraction protocol, for total phenolic content (TPC) and proanthocyanidins (PA) of grape seeds and seed press cake from three different Oklahoma grown grape varieties (‘Merlot’, ‘Cabernet Sauvignon’ and ‘Sangiovese’). The influence of different extraction factors: solvents, extraction time and pH of extraction solvents on TPC and PA extraction yield were assessed. Ultrasound-assisted extraction was also evaluated in terms of its capacity to increase the solvent potential of acidified water. Quantification of TPC and PA was done using a Folin-Ciocalteau spectrophotometric micro method and vanillin colorimetric method, respectively. Solvents adjusted at pH 2 favored TPC and PA extraction yield compared to pH 3 with most solvents. Proanthocyanidin extraction yield was probably insensitive to pH with 80% acetone and water as extraction solvents. Extraction time of 6 h was probably needed for maximum recovery of TPC from grape seeds and press cake, whereas PA yield might not increase with extraction time above 3 h. The optimal solvent for TPC from both seed and press cake appeared to be 70% ethanol at pH 2, while PA extraction was mostly favored by 80% acetone adjusted at pH 2. For 6 h of extraction, water at pH 2 recovered about 20% of TPC compared to 70% ethanol at pH 2 and about 35% of PA compared to 80% acetone at pH 2. Ultrasound-assisted extraction with water significantly reduced TPC and PA extraction time to 10 minutes and increased TPC and PA recovery about 10% compared to conventional extraction with water for 1 h. Using optimum extraction conditions TPC ranged from 8.3% to 9.4% in seeds and 8.4% to 12.1% in press cake and PA ranged from 4.3% to 6.8% in seeds and 4.1% to 5.6% in press cake on a dry, oil-free basis.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Plant phenolics have attracted increased interest of researchers due to their potential health benefits. Plant phenolic compounds have antioxidant properties and act as free radical scavengers, regulate nitric oxide, decrease leukocyte immobilization, inhibit cell proliferation and exhibit phytoestrogenic activity protecting the human body against different diseases (Arts and Hollman, 2005; Brigitte et al., 2005). Polyphenols have the capacity of scavenging the reactive oxygen species (ROS) such as radical and non-radical oxygen species and oxidatively generated free radicals which are deleterious to human health (Quideau et al., 2011). Phenolic compounds also have potential uses in the food industry. Phenolic compounds have been reported to prevent food degradation and increase the quality of food because of their antibacterial property (Jayaprakasha et al., 2001; Maqsood et al., 2013; Mastromatteo et al., 2010).

Plant phenolics are secondary metabolites found naturally in different plants such as fruits, vegetables, cereals and beverages (Arts and Hollman, 2005; Brigitte et al., 2005). These bioactive compounds scavenge the reactive oxygen species produced in plants under environmental stress and pathogenic attack, activating the plant defense mechanism (Lattanzio, 2013; Treutter, 2006).

Polyphenols mainly include phenolic acids, flavonoids, stilbenes and lignans (Pandey and Rizvi, 2009). Among the different classes of polyphenols, the proanthocyanidins (condensed tannins), the gallo- and ellagitannins (hydrolysable tannins) and the phlorotannins are considered as true polyphenols (Quideau et al., 2011).

Grapes (*Vitis vinifera* L.) are a good source of polyphenols. However, the variety of grapes, climatic conditions, site of production and degree of maturity influence the phenolic profile in grape (Revilla et al., 1995). Grape pomace, a solid waste obtained after pressing whole grapes for juice or wines, contains skins, stems and seeds and represents 20% (w/w) of the total grape fresh weight used for wine production (Panouillé et al., 2007). A considerable amount of polyphenols are retained in these wastes. Grape seeds represent 60% of total polyphenols in the grape and contains about 5%-8% polyphenols by weight depending on the variety (Shi et al., 2003b). A major portion (50% - 70%) of proanthocyanidin in grape is contained in grape seed (Freitas et al., 1998; Mylonaki et al., 2008). A considerable amount of total polyphenols is retained in grape seed press cake after oil expression although some polyphenols suffer thermal degradation during the pressing operation, which could change the polyphenolic profile (Maier et al., 2009). Extraction, purification and characterization of individual phenolic components have been accomplished in a wide variety of plants. Cost-effective recovery and replacement of the extraction solvents unfit for human consumption with generally recognized as safe solvents such as water are the major concerns in commercial extraction of phenolic compounds.

Selection of the optimum extraction condition of plant phenolics is a challenging process. Recovery of phenolic compounds has been reported to be influenced mainly by their chemical nature in the plant material, the extraction method, solvent type, sample particle size, extraction time, storage time, temperature and presence of interfering substances in the matrix (Corrales et al., 2009; Ignat et al., 2011). Published literature is not conclusive about any ideal extraction solvent for phenolic compounds and different mixtures have been proposed with variable extraction efficiencies (Cheng et al., 2012; Fontana et al., 2013). In addition, the commonly used organic extraction solvents such as methanol, ethanol and acetone are expensive and difficult to recover from the phenolic extracts to make the extracts suitable for human consumption. Increasing extraction potential of human health friendly extraction solvents such as water could decrease the extraction cost of plant phenolics.

Solid-liquid extraction (SLE), the most common method reported for polyphenols extraction from grape seeds, is sometimes assisted with other technologies such as microwave, enzyme and ultrasound to increase the extraction efficiency (Fontana et al., 2013; Liu and White, 2012). Ultrasound-assisted extraction (UAE) is one of the emerging technologies that have been reported to promote SLE kinetics by increasing the recovery and/or by shortening the extraction time. The use of UAE to increase the extraction potential of water and accelerate the polyphenol extraction rate was an important aspect of this study.

There is limited information on the polyphenolic content in the seeds of grape varieties grown in Oklahoma. The three Oklahoma grown grape varieties used in this

study were ‘Merlot’, ‘Cabernet Sauvignon’ and ‘Sangiovese’. Seeds from these grape varieties grown in Siberia have been reported to have 77 mg GAE/g, 69.57 mg GAE/g and 47.38 mg GAE/g total phenolic content on a dry weight basis (Pantelic et al., 2016). In another study, ‘Merlot’ and ‘Cabernet Sauvignon’ seeds grown in Turkey were found to have 105.7 mg GAE/g and 103.7 mg GAE/g of total phenolic content, respectively, on a dry weight basis. The study also reported 33.1 mg/g and 29.4 mg/g total polymeric proanthocyanidin, respectively, from ‘Merlot’ and ‘Cabernet Sauvignon’ seeds (Bozan et al., 2008).

Lack of standard and optimum polyphenol extraction protocol, limited information on the polyphenolic content of Oklahoma grown grape varieties and potential use of ultrasound to improve polyphenol extraction were the main reasons behind this study. The objectives of this study were to:

- Optimize the extraction of proanthocyanidins from grape seeds and grape seed press cake.
- Quantify the total polyphenols and proanthocyanidins from grape seed and press cake of Oklahoma grown grape varieties.
- Evaluate the performance of ultrasound assisted polyphenol extraction in decreasing time and/or increasing extraction yield using water as extraction solvent.

CHAPTER II

REVIEW OF LITERATURE

2.1 Phenolic compounds

There is a broad diversity of phenolic compounds with more than 8,000 compounds identified so far in various plant species. Plant polyphenols have been primarily linked to defense against environmental stress and pathogenic aggression (Bavaresco, 2003; Lattanzio, 2013; Treutter, 2006). Plant phenolic compounds are naturally occurring secondary metabolites that regulate plant responses to stress (Treutter, 2006) and contribute to color, flavor, odor, astringency, bitterness and other sensory and nutritional qualities of foods (Ignat et al., 2011; Pandey and Rizvi, 2009). Phenolic compounds are divided into different types based on their chemical structure (Fig.1).

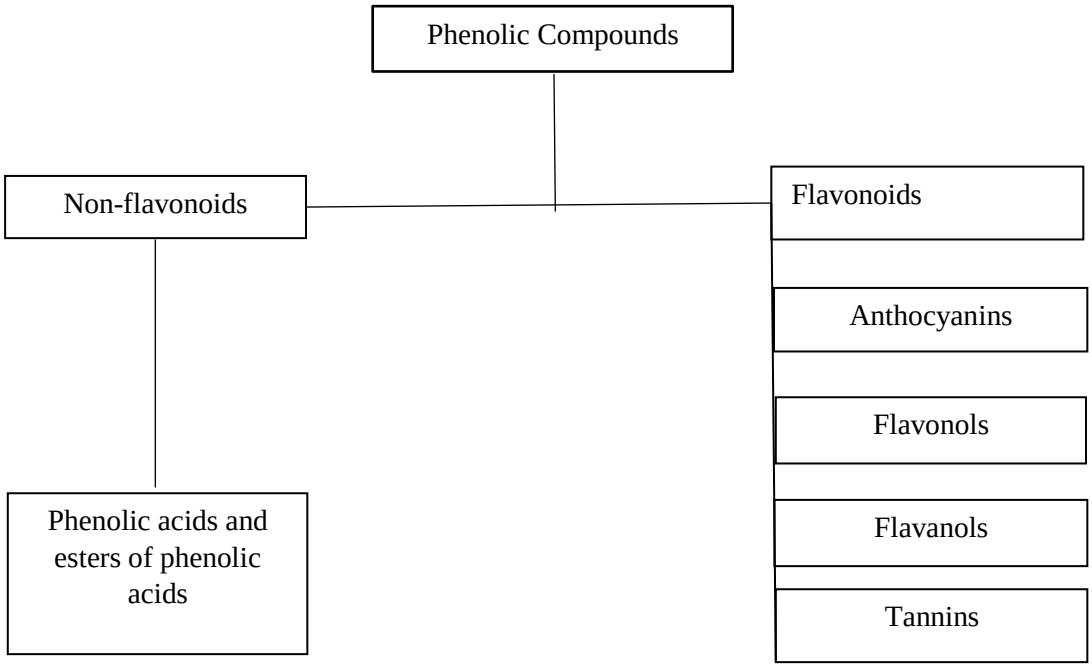

Fig 1: Classification of phenolic compounds based on their chemical structure (Spencer et al., 2008)

Different classes of phenolic compounds are discussed below:

2.1.1 *Phenolic acids*

Phenolic acids can be divided into two classes: derivatives of cinnamic acid or of benzoic acid based on the number and position of hydroxyl groups on the aromatic ring (Pandey and Rizvi, 2009; Robbins, 2003). Only a small fraction of phenolic acids are free in higher plants, the rest of the phenolic acids are found combined as esters and glycosides (Nagels et al., 1980; Robbins, 2003). The most common phenolic acids found in grapes are hydroxycinnamic acids (p-coumaric acid, ferulic acid, sinapic acid and caffeic acid) and hydroxybenzoic acids (gallic acid, protocateic acid and syringic acid) (Fig. 2). Esters of phenolic acids such as caftaric acid, coutraic acid and trans-fertaric

acid are also commonly found in grapes (Macheix and Fleuriet, 1990). The degree of degradation susceptibility and the extractability of phenolic compounds vary with the forms of phenolic acids (Ross et al., 2009).

Fig. 2. Chemical structures of hydroxy-cinnamic acid (Left) and hydroxybenzoic acid (Right) (Pandey and Rizvi, 2009)

2.1.2 *Flavonoids*

Flavonoids are an extensively studied group of polyphenols. More than 4,000 types of flavonoids, divided into six subclasses: (flavonols, flavanones, flavones, flavanols, anthocyanin and isoflavones) have been identified so far (Spencer et al., 2008). "Flavonoids are low molecular weight compounds with the basic chemical structure of C_{15} (C_6-C_3-C_6), essentially composed of two aromatic rings linked by a 3-carbon bridge" (Merken and Beecher, 2000) as shown in fig. 3.

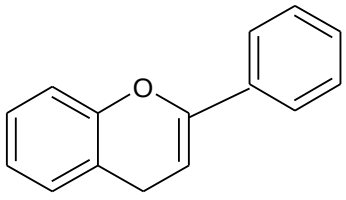

Fig 3. Basic monomeric structure of flavonoids (Pandey and Rizvi, 2009)

Flavanols are commonly referred as catechins. Catechin and epicatechin are examples of monomeric flavanols (Tsao, 2010). Flavanols can be found in their oligomeric and polymeric forms, commonly referred to as condensed tannins or proanthocyanidins (Cheynier, 2005; Tsao, 2010). Chemical structure of catechin (Savova et al., 2007) is shown in fig. 4.

.

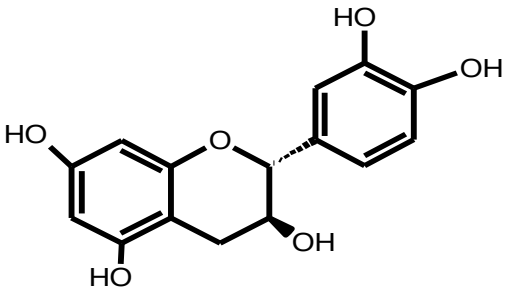

Fig. 4. Chemical structure of catechin (Savova et al., 2007)

2.1.3 *Anthocyanins*

Anthocyanins, usually glycosylated, are water soluble pigments found in the vacuoles and exhibit different colors as red, purple, or blue depending on pH. They have potential use as food and beverage colorants and have exhibited health benefits (Konczaka and Zhang, 2004). "The basic structure of anthocyanin is anthocyanidin which consists of an aromatic ring A bonded to an heterocyclic ring C that contains oxygen, which is also bonded by a C-C bond to a third aromatic ring B" (Konczaka and Zhang, 2004; Fig. 5). Six common anthocyanins that contribute to pigmentation are: Malvidin 3-O-Glu, Delphinidin 3-O-Glu, Peonidin 3-O-Glu, Cyanidin 3-O-Glu, Petunidin 3-O-Glu and Pelargonidin 3-O-Glu (Gao and Mazza, 1995).

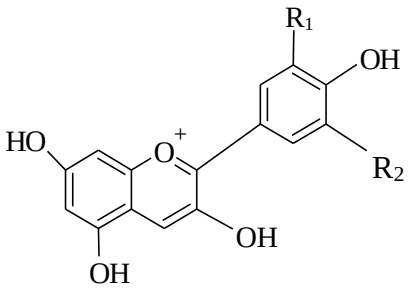

Figure 5. Chemical structure of anthocyanins (Pandey and Rizvi, 2009)

2.1.4 *Stilbenes and lignans*

Stilbenes are found in grape berry skins (Shi et al., 2016). Stilbenes are produced in plants in response to environmental stress and pathogenic attack (Bavaresco, 2003). A major stilbene found in grapes is resveratrol (Fig. 6). Chemical structure of stilbenes contains two phenyl moieties connected by a two-carbon methylene bridge (Pandey and Rizvi, 2009).

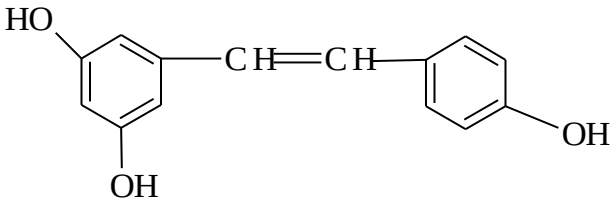

Fig 6. Chemical structure of resveratrol (Ignat et al., 2011)

Lignans are formed due to the oxidative dimerization of two phenylpropane units (Fig. 7). Potential applications of lignans and their synthetic derivatives in cancer chemotherapy have drawn considerable interest from researchers (Saleem et al., 2005). Although some lignans are thought to play a role in plant defense mechanism because of

tantimicrobial, antifungal, antiviral, antioxidant, insecticidal and antifeeding properties, their actual biological functions in plants is still unclear (Saleem et al., 2005).

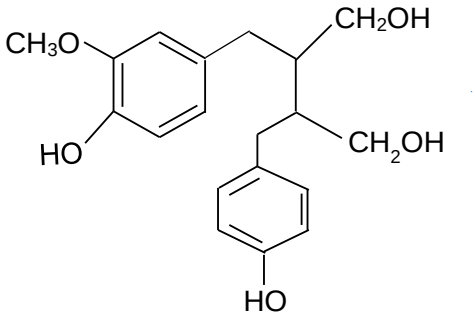

Fig 7. Chemical structure of lignan (Pandey and Rizvi, 2009)

2.1.5 *Tannin*

Tannins are relatively high molecular weight phenolic compounds and can be divided into hydrolysable and condensed tannins (Porter et al., 1989). Proanthocyanidins are condensed tannins which impart astringency and bitterness in food, but also exhibit potential health benefits (De Sa et al., 2014). Proanthocyanidins are derived from the oligomerization of flavan-3-ol units such as epicatechin, epigallocatechin and fisetinidol (Quideau et al., 2011) and contain diphenylpropane structure of C_6-C_3-C_6) (Liu and White, 2012).

Proanthocyanidin varies in size from a monomer to oligomers with 4 or more units to as large as 20 units or more depending on the degree of polymerization (Fine, 2000; Fig. 8). The degree of polymerization is also associated with the degree of astringency; oligomeric proanthocyanidins being less astringent than polymeric proanthocyanidins (Da Silva et al., 1991; Vidal et al., 2003).

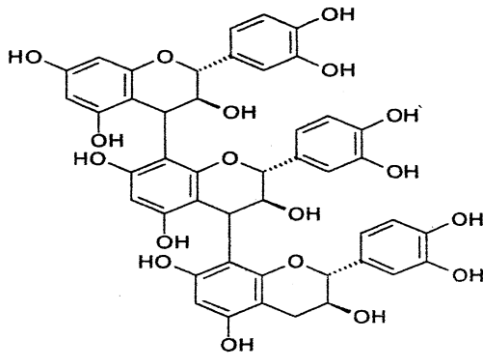

Fig 8. Chemical structure of pronathocyanidins (Scalbert et al., 2005)

2.2 Biosynthesis of phenolic compounds in plants

Shikimate/phenylpropanoid pathway or the “polykende” acetate/malonate pathways are the major biosynthetic pathways of phenolic compounds (Chapman and Ragan, 1980; Croteau et al., 2000; Fig. 9). Endogenous control of phenolic compound synthesis in plants occurs during plant development and differentiation (Haddock et al., 1982; Strube et al., 1993), whereas biotic factors such as insects, pathogens and abiotic factors such as different environmental stresses exogenously control the phenolic compound synthesis. Environmental stresses and pathogenic aggression promotes the synthesis of phenolic compounds to strengthen the defense mechanism (Bennett and Wallsgrove, 1994). Moreover, phenolic compounds also contribute as a signaling compound to attract pollinators and protect the plants from ultraviolet radiation (Lattanzio, 2013).

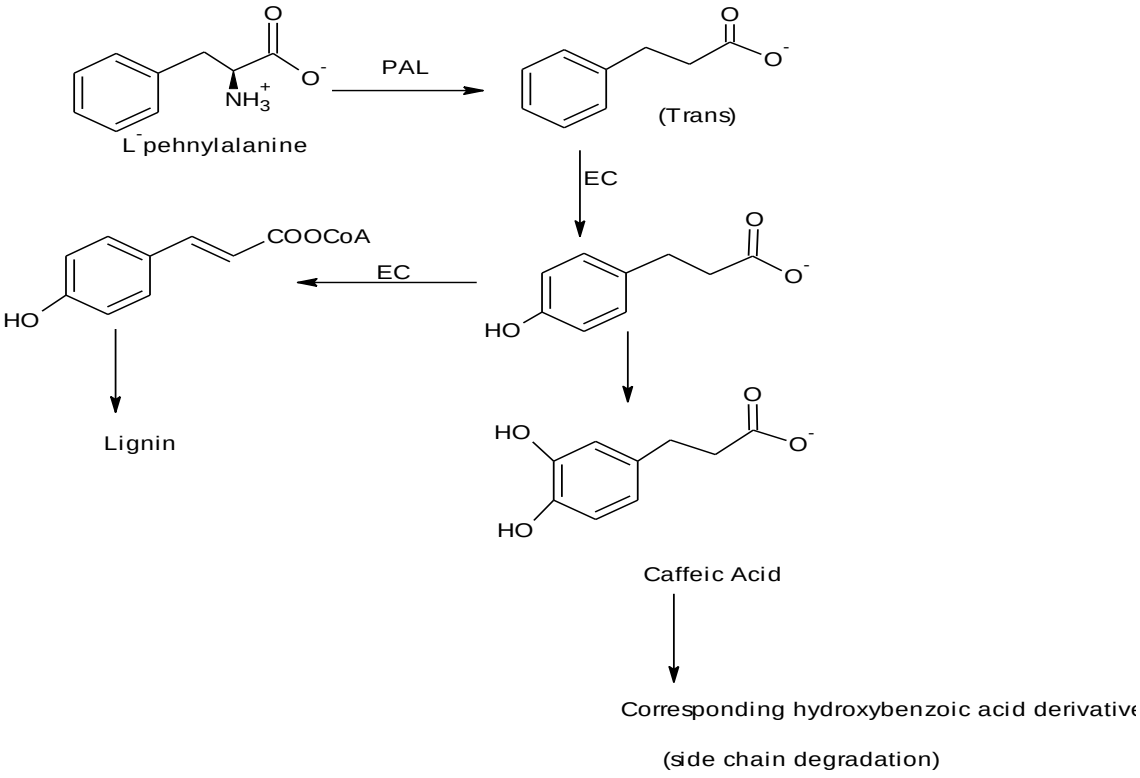

Fig 9. Biosynthetic pathways of hydroxycinnamic and hydroxybenzoic acids from L-phenilalanine (Robbins 2003).

2.3 Winery wastes

A substantial amount of wastes are produced from wineries each year. Waste management has become a challenge for wineries due to the higher cost of waste treatment and environmental hazards associated with winery waste (Musee et al., 2007). Although winery wastes have been used as fertilizer and livestock feed (Arvanitoyannis et al., 2006; Sri Harsha et al., 2013), phytotoxicity of phenolic compounds inhibiting seed germination is a serious concern (Northup et al., 1998). However, extraction of phenolic compounds from winery waste can add value to the winery byproducts. Byproducts

during winemaking processes are grape pomace, lees, stalk and wastewater sludge accounting for 62%, 14%, 12%, and 12% of total winery wastes, respectively (Ruggieri et al., 2009; Fig. 10).

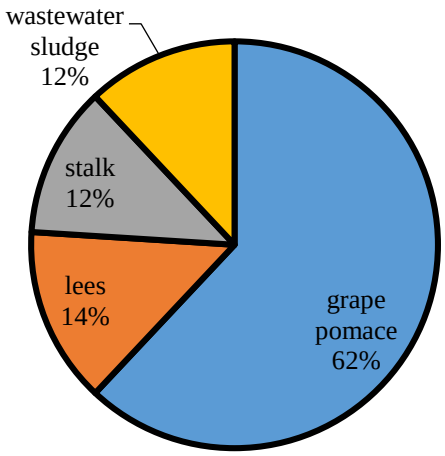

Fig 10. Different winery byproducts (Ruggieri et al., 2009)

These byproducts are produced at different stages of the wine making process depending on whether a red wine (fermentation includes seeds and skins) or white wine (fermentation does not include seeds and skins) is being produced. Typical byproducts produced by either style of wine making are shown in fig.11.

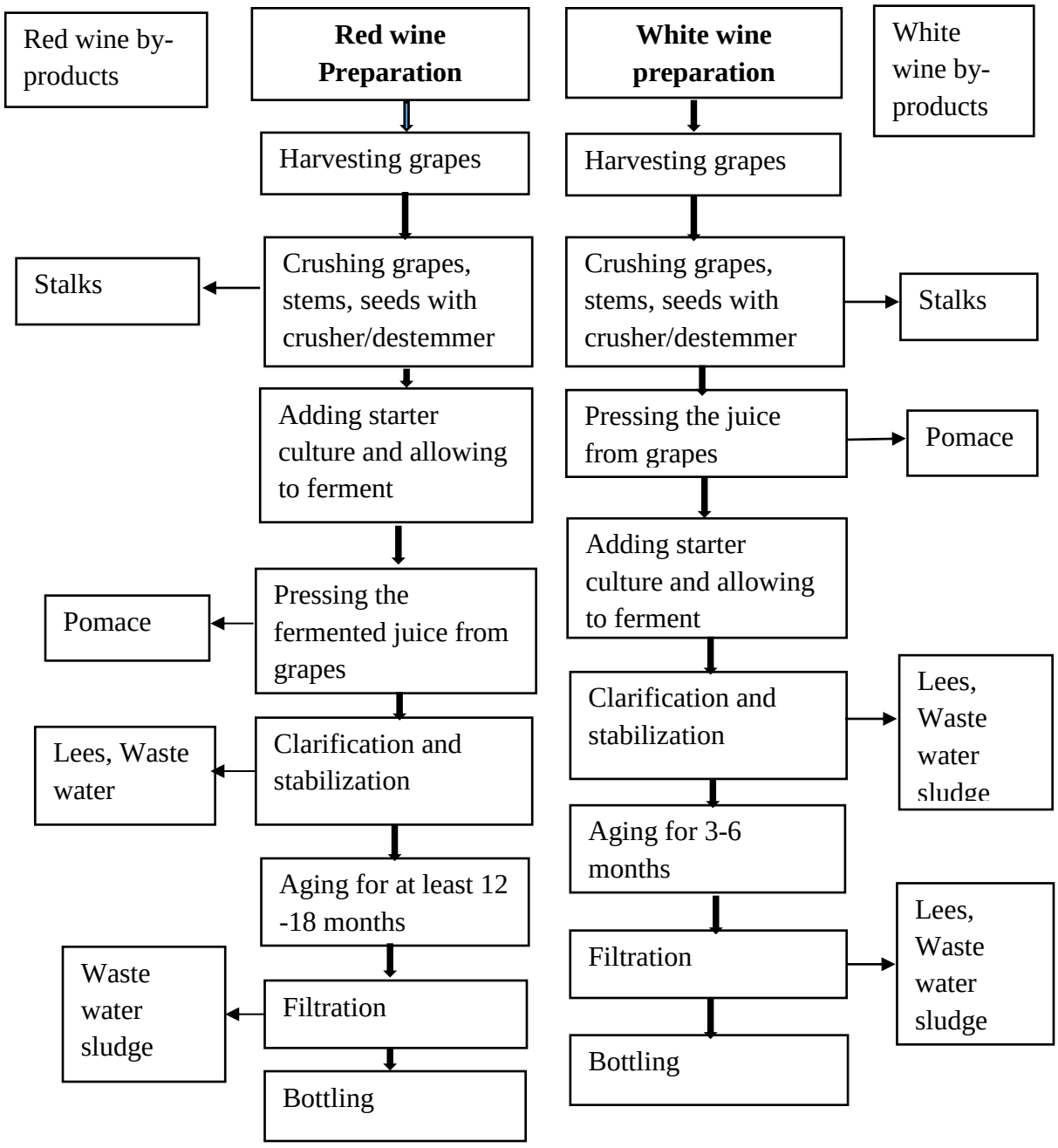

Figure 11. Flow chart of winery waste production during wine making (Thunga, 2015)

2.3.1 *Grape pomace*

Grape pomace, solid residue after pressing grapes for juice and wines, has been suggested as a cheap source of polyphenols (Paramas et al., 2004). The components of grape pomace are grape seeds, skins and pulp and stems representing 30%, 40% and 30% of total pomace on a wet basis, respectively (Göhl, 1982).

2.3.2 *Grape seeds*

Approximately 32% to 52% of grape pomace is represented by grape seeds on a dry weight basis (Schieber et al., 2002). Grape seeds are composed of up to 40% fiber, 16% oil, 11% protein, 7% phenolic compounds and other substances (De Campos et al., 2008). Generally, grape seeds are reported to have 5% - 8% of polyphenols by dry weight (Shi et al., 2003b). However, total polyphenolic content in grape seed may reach up to 13.8% on a dry weight basis (Makris et al., 2007). Various studies have suggested that the grape seed press cake, the residue after extracting oil from grape seed, is a good source of polyphenols although high temperature exposure during grape pressing for oil extraction could degrade polyphenols resulting in a change in total polyphenolic content and polyphenolic profile (Maier et al., 2009).

2.4 Distribution of polyphenols in grape seeds

There is an uneven distribution of polyphenols in the grape berry. Grape seed constitutes 60% to 70% of total extractable phenolics present in grape berry, whereas skin and pulp contain 28% to 35% and 10% or less extractable phenolics, respectively. The predominant phenolic compounds in grape seeds are proanthocyanidin, which are mostly located in the outer soft coat of the seed (Thorngate and Singleton, 1994), and falvan-3-

ols including catechins (catechin, epicatechin and procyanidins) and their polymers (Prieur et al., 1994; Da Silva et al., 1991; Godjevac et al., 2010). The procyanidins represent a major part (50% - 70%) of total polyphenols in grape seed (Mylonaki et al., 2008).

2.5 Potential health benefits of grape seed polyphenols

An abundance of information on the beneficial properties of plant phenolics has caused increased awareness of the general public and raised the keen interest of researchers on the potential health benefits of polyphenols. Regular consumption of a diet rich in plant polyphenols has been reported to limit the development of diseases such as arthritis, dementia, cardiovascular illness, diabetes, osteoporosis, cancer and neurodegenerative diseases (Mennen et al., 2004; Scalbert et al., 2005; Sun et al., 2002). Polyphenols act as free radical scavengers, regulate nitric oxide, decrease leukocyte immobilization, inhibit cell proliferation and exhibit phytoestrogenic activity protecting the human body against different diseases (Arts and Hollman, 2005; Brigitte et al., 2005). Polyphenols have the capacity of scavenging the reactive oxygen species (ROS) such as radical and non-radical oxygen species and oxidatively generated free radicals which are deleterious to human health (Quideau et al., 2011). The phenol functional group of polyphenols is thought to prevent chronic human diseases by donating a hydrogen atom to a free radical produced during lipid autoxidation thus breaking the chain of oxidation in the cells (Quideau et al., 2011). Grape seed polyphenols have also been reported to exhibit different therapeutic properties such as: antibacterial, antiviral, anti-inflammatory, anti-allergic and vasodilatory actions (Fine, 2000). Proanathocyanidin rich grape seed extract has been reported to be safe to be used in foods (Yamakoshi et al., 2002). Furthermore, Procyanidin oligomers

present in grape seeds are reported to be 20 times more efficient than vitamin C and 50 times more efficient than vitamin E as antioxidants (Uchida, 1980).

2.6 Extraction of polyphenols from grape seeds

Extraction method/technique is an important step for the isolation, identification and purification of phenolic compounds. There is not any established standard extraction method and different extraction methods have been used for the phenolic compound extraction from grape seeds (Fontana et al., 2013). The Solid-Liquid Extraction (SLE) method is the most commonly used polyphenol extraction method from grape seeds (Fontana et al., 2013; Liu and White, 2012). Solid-liquid extraction is a phenomenon in which analytes in the sample matrix diffuse to the solvent when they come in contact with the solvent (Fontana et al., 2013). Solid-liquid extraction sometimes is assisted by other methods such as microwave (Li et al., 2011), ultrasound (Carrera et al., 2012) and some others to improve the extraction. Extraction efficiency and recovery of phenolic compounds during SLE is influenced by particle size, solid to liquid ratio, extraction solvent, extraction time, extraction temperature and solvent pH (Fontana et al., 2013). Some important factors impacting polyphenol extraction are discussed below:

2.6.1 *Extraction solvent*

Polyphenols are easily soluble in polar or semi polar media due to their polar nature. Aqueous organic solvents have been widely used because the permeability of cell tissue is increased due to the presence of water, which enables better mass transfer by molecular diffusion (Cheng et al., 2012; Jayaprakasha et al., 2001). Organic solvents such as ethanol, methanol, acetone and ethyl acetate have been mostly used for polyphenol

extraction (Fontana et al., 2013). However, those solvents demonstrated variable extraction efficiencies depending on other extraction variables considered (Cheng et al., 2012). Aqueous ethanol has been found to give higher yield of phenolic compounds than pure ethanol (Maier et al., 2009; Shi et al., 2003a; Yilmaz and Toledo, 2006). Different studies have recommended that different concentrations of ethanol could be used for the extraction of phenolic compounds from grape seeds and grape seed press cake. According to Shi et al., (2003a) and Yilmaz and Toledo (2006), 50% ethanol is a better solvent for phenolic compound extraction compared to other higher or lower ethanol concentrations. Nonetheless, 75% aqueous ethanol was found to give an optimal yield of phenolic compounds from grape seed press cake (Maier et al., 2009). According to Fontana et al. (2013), ethanol/water mixtures are relatively better for the extraction of total polyphenolic content compared to acetone and methanol/water mixtures. Even the results from different studies contradict each other. Some studies have suggested that acetone/water mixtures give better yield for proanthocyanidins extraction from grape seeds (Yilmaz and Toledo, 2006), whereas other studies have demonstrated higher extraction efficiency of ethanol/water mixtures (Torres et al., 2002). It is fair to say that research studies are not conclusive about the ideal extraction solvent for polyphenols.

2.6.2 *Solvent pH*

The use of acidified extraction solvent could increase recoveries of phenolic compounds, which are affected by acidity of extraction solvent (Fontana et al., 2013). Total phenolic content (TPC) was increased 3-fold when hydrochloric acid was added to the extraction solvent (Vatai et al., 2009). However, concentration of the solvent (ethanol) was found to interact with the solvent pH in determining the polyphenolic yield.

A basic pH of the extraction solvent with low ethanol concentration was demonstrated to give a better yield of polyphenols from grape pomace, whereas acidic pH was found to give better yield with high ethanol percentage (Librán et al., 2013). Similarly, higher pH of extraction solvent (ethanol) with higher ethanol concentration decreased the recovery of total polyphenols from grape stems. The study further demonstrated that the effect of solvent pH was different for stems from different grape varieties (Karvela et al., 2009a). Similarly, the use of a low pH extraction solvent (ethanol) with prolonged extraction period was suggested to give a higher yield of proanthocyanidins from grape seeds (Karvela et al., 2009b).

2.6.3 *Extraction time*

Extraction time has been identified as an important factor in phenolic compound extraction. According to Spigno et al. (2007), a longer extraction time (> 8 h) at a lower extraction temperature of 45 ^{0}C compared to 60 ^{0}C gives higher phenolic yield. However, extraction for shorter times (< 8 h) at the higher extraction temperature of 60 ^{0}C was economically feasible using aqueous ethanol as an extraction solvent. Several studies have demonstrated that although optimal reaction time for proanthocyanidin extraction from grape seeds using ethyl: acetate water mixture was 24 h, a plateau was reached after 15 hours (Liu and White, 2012). The extraction time of polyphenols has been found to be influenced by sample particle size, solvent pH and extraction temperature. Shi et al. (2003a) found 1.5 hours as an optimum time for polyphenol extraction from grape seeds using 50% ethanol at 65 ^{0}C. Huh et al. (2004) reported that 8 h of extraction at 70 ^{0}C using 70% ethanol was an optimal condition for the extraction of oligomeric proanthocyanidins from wild grape seeds. It was also observed that a smaller particle size

increases the rate of proanthocyanidin extraction compared to larger particle size of sample (Pekić et al., 1998). Variations in the polyphenol extraction time have been found in many studies depending on other extraction factors.

2.6.4 *Extraction temperature*

Extraction temperature is another extraction variable that has significant influence on phenolic compounds extraction (Bucić-Kojić et al., 2007). Generally increase in extraction temperature up to 80 ^{0}C has shown an increased yield of phenolic compounds (Bucić-Kojić et al., 2007; Ju and Howard, 2003; Shi et al., 2003a; Spigno et al., 2007). The upper limit for the extraction temperature appears to be limited by possible degradation of phenolic compounds due to the oxidation and decomposition of the desired compounds at higher extraction temperature (Shi et al., 2003a). Higher extraction temperature above 100 ^{0}C was found to have a degradative effect on catechins and epicatechin (Palma et al., 2001), and anthocyanins (Ju and Howard, 2003). Extraction temperature of 80 ^{0}C has been reported to maintain the phenolic compound stability (Ju and Howard, 2003).

Extraction temperature could have an effect on individual phenolic compounds differently. According to Bucić-Kojić et al. (2007), extraction temperature of 80 ^{0}C with 50% aqueous ethanol gave a better yield compared to a lower temperature. Similarly higher extraction temperature of 70 ^{0}C was found to increase the oligomeric proanthocyanidin yield from wild grape seeds (Huh et al., 2004).

2.7 Ultrasound assisted Solid Liquid Extraction

Ultrasound technology is one of the emerging technologies that has received focus during the past few years, especially in the food industry. Ultrasound consists of mechanical waves which cause oscillation of matter during energy transfer, at a frequency at or above 20 kHz (Soria and Villamiel, 2010). There are two types of ultrasonic waves; high frequency (100 kHz to 1 MHZ) also called low power ultrasound (< 1 W cm^{-2}) and low frequency (16 kHz to 100 kHz) also referred as high power ultrasound ($10 – 1000$ W cm^{-2}). These two kinds of ultrasound have their own specific uses. Low-frequency ultrasound is used for non-destructive analysis such as quality assessment, whereas high-frequency ultrasound is used to alter physical or chemical properties of the food. Assessment of firmness, ripeness, sugar content and acidity are some common uses of low power ultrasound (Soria and Villamiel, 2010). High-intensity ultrasound has been used to disrupt cells, generate emulsions, and disperse aggregated materials (McClements, 1995). High power ultrasound has also been used for extraction purpose. Application of ultrasound is not limited to the food industry. Ultrasound has been used in aquaculture to increase egg hatching and survival of fingerlings. Ultrasound has also been reported to stimulate seed germination by breaking dormancy in beans and rice (Mason et al., 1996).

Ultrasound has been successfully used to either increase recovery and/or shorten the extraction time during extraction of bioactive compounds in comparison to conventional extraction methods (Carrera et al., 2012; Khan et al., 2010; Pan et al., 2012). Other benefits of ultrasound assisted extraction are less solvent consumption and increased quality of the extracts (Wang and Weller, 2006). Cavitation phenomenon

during ultrasound assisted extraction process has been attributed to increased polyphenolic yield and reduced time of extraction (Mason et al., 1996). When sufficiently high power ultrasound is supplied to an extraction solvent, cavitation bubbles form from the gas nuclei in the liquid. These cavitation bubbles grow over time and collapse violently generating very high local temperature (5000 K) and extreme pressure (1000 atm) once the critical size is reached resulting in the high sheer energy waves and turbulence in the cavitation zone (Soria and Villamiel, 2010). This causes better solvent penetration into the cells and tissue, efficient cell disruption and enhanced mass transfer (Mason et al., 1996).

The extent of cavitation during extraction is also dependent on several other factors such as energy, intensity, medium surface tension, vapor pressure, nature and concentration of dissolved gases and the pressure of the treatment (Soria and Villamiel, 2010). Furthermore, studies have reported the potential use of ultrasound to increase the extraction potential of GRAS solvents such as water (Vilkhu et al., 2008).

Ultrasound-assisted extraction has been reported to increase polyphenolic yield up from 6% to 35% from different plant tissues including grape pomace (Vilkhu et al., 2008). Extraction time was significantly reduced to 6 min using ultrasound-assisted extraction of phenolic compounds from grapes compared to the classical method for 1 hour using 50% ethanol as an extraction solvent. The recovery of phenolic compounds was either similar to or higher than the classical extraction method (Carrera et al., 2012). The study also demonstrated that 10 ^{0}C of extraction temperature gave a higher yield of total phenolics and anthocyanin compared to other levels of extraction temperature (10 ^{0}C, 20 ^{0}C, 50 ^{0}C, and 75 ^{0}C). Although phenolic recovery is supposed to increase with

increased extraction temperature, decrease in the yield above 10 ^{0}C was observed which could be because of oxidative degradation reaction promoted by higher temperature. No significant difference in the yield was found between amplitude 50% and 100% (Carrera et al., 2012). Using a response surface methodology (RSM), optimum conditions for ultrasound-assisted extraction of phenolic compounds from grape seeds were found to be 53.15% ethanol, 56.03 ^{0}C temperature and 29.03 min extraction time (Ghafoor et al., 2009). An increase in 30% total phenolic yield from apple pomace using ultrasound assisted extraction with water was reported at 40 ^{0}C compared to conventional extraction and the higher antioxidant property was found in the extract obtained using ultrasound assisted extraction (Pingret et al., 2012). The ultrasound assisted solid-liquid extraction could be used to increase the extraction potential of cheap, environmentally friendly and healthy solvents such as water. Ultrasound assisted extraction has shown potential to benefit commercial extraction of bioactive compounds from winery wastes. This study also investigated the performance of ultrasound assisted extraction with water to explore its commercial applications.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals

Analytical grade chemicals and reagents were used in this study. Acetone (99.5 %), ethanol (95%) and concentrated hydrochloric acid were obtained from Pharmaco - AAPER (Shelbyville, KY). Gallic acid, Dimethylacetamide (DMAC) and Folin-Ciocalteau phenol reagent was purchased from Sigma-Aldrich (St. Louis, MO). Sodium carbonate, sodium hydroxide and diethyl ether was obtained from Fisher Scientific (Hampton, NH). Proanthocyanidin B_2 was obtained from ChromaDex (Irvine, CA). Buffer solutions for pH measurement (7.01 and 4.01) were obtained from Hanna Instruments (Woonsocket, RI).

3.2 Pomace Seed Separation

Fresh grape pomace of three different varieties (‘Merlot’, ‘Cabernet Sauvignon’ and ‘Sangiovese’), were supplied by Canadian River Winery (Lexington, OK) and were processed at Redbud Farms (Washington, OK) in September, 2015. Pomace was initially separated into seed, stem and skin using a modified trommel cleaner (Fig. 12).

Pomace was added to the pomace breaker/feeder mechanism and any clumps were broken apart by a rotating finger mechanism prior to dispersal into the trommel drum. The trommel drum was 0.91 m (3 ft) in diameter and 1.82 m (6 ft) in length and was covered with hardware cloth with 0.64 cm (0.25 inch) square openings. The trommel drum was continuously rotated with a variable speed electric motor at a relative speed setting of 8 out of 10. Pomace was fed at a rate of 2.27 kg (5 lbs) per minute. Trommel downwards angle was maintained at 9 to 12 degrees. Seeds and small pieces of grape skin and stems exited though the hardware cloth and were collected in bins below the cleaner. Large skin and stems passed through the trommel, exited through a discharge chute and were discarded. The separated seed fraction was then air dried to lower than 11% moisture and transported from Washington, OK to Stillwater, OK for further cleaning and processing at the Robert M. Kerr Food and Agricultural Products Center.

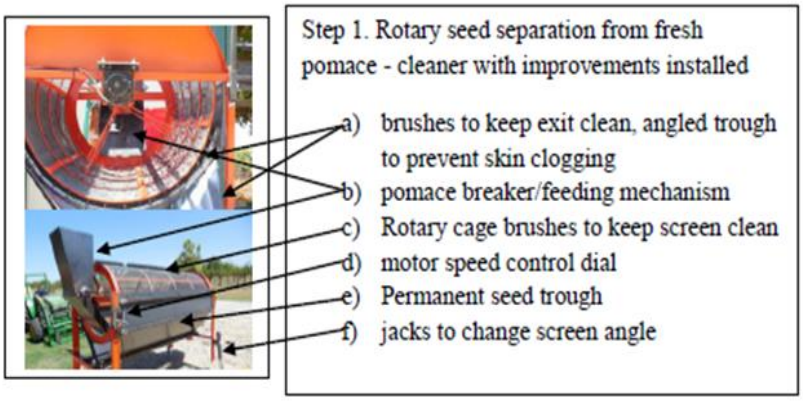

Fig 12. Modified seed trommel cleaner

3.3 Seed Cleaning

The air-dried seed fraction from the initial trommel separation contained substantial quantities of contaminating small skins and stems (20% to 30%; w/w) which was removed using a Ferrell Eclipse 324 vibratory seed cleaner (A.T Ferrell Company, Bluffton, IN) shown in Fig.13. A trough at the top of the cleaner metered seed into the cleaner at a rate of 0.9 to 1.4 kg min^{-1} (2 to 3 lbs min^{-1}). Seeds first passed through a 5 mm (diameter) round-holed screen to separate large debris and then passed over a 4 mm (diameter) round-holed screen to separate small (mostly immature) seeds and finally over a 3 mm (diameter) round-holed screen to separate very small debris. Cleaned seed (collected at the back of the cleaner and representing 70% to 80% of the initial air dried trommel cleaner seed fraction) was utilized for oil pressing.

Fig. 13. Eclipse 324 seed cleaner

3.4 Seed Oil Pressing

Immediately prior to oil pressing, seed moisture content was determined with a Case-IH MT-16 Moisture Meter (Case IH, Inc., Racine, WI). The meter's "Arbitrary

Response" reading was calibrated against actual moisture content for grape seed of numerous varieties and at differing moisture contents. The calibration curve shown in Fig. 14 was used for converting seed "Arbitrary Response" readings into seed moisture content. If seed were below 12% moisture (meter reading above 1860), seeds were rehydrated by applying water of a weight sufficient to raise their moisture content to 12 %, completely mixing and allowing seed to equilibrate at room temperature for at least 3 h. Multiple re-moistening episodes were at times needed to achieve a stable moisture content within the desired range due to water evaporation from the surface prior to seed water uptake, especially if seed were below 7% moisture prior to rehydration. Seed re-hydration was necessary to prevent oilseed press barrel clogging during the seed pressing operation.

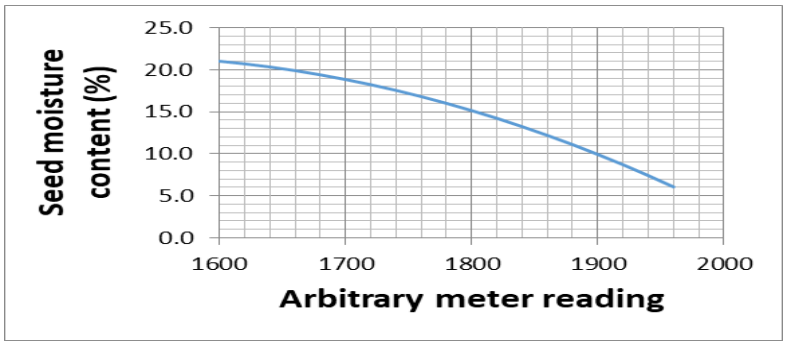

Fig. 14. Case-IH moisture meter calibration chart

Separated seeds were pressed using a Tokul-Agro oilseed press (Tokul-Tarim Co. Ltd., Izmir, Turkey) using a cross-head speed of 60 rpm and an outlet dye size of 4 mm (Fig. 15). Temperature of the oilseed press barrel was maintained at 80 °C with a heating collar. Seeds were processed in 4.55 kg batches (10 lbs) by loading a funnel affixed to the mill; oil pressing rate averaged 0.09 kg min^{-1} (0.2 lbs min^{-1}). Oil dripping from the oil press barrel was collected into a tared container and presscake exiting from the oilseed

press dye as a ribbon was collected into a tared plastic tub. Oil press oil yields (w/w) were 6.2% for ‘Merlot’, 7.2% for ‘Cabernet Sauvignon’ and 7.8% for ‘Sangiovese’. Cleaned seeds and press cake were collected into labeled air tight plastic bags and stored in room temperature to await phenolic extraction trials.

Fig. 15. Tokul-Agro oilseed press

3.5 Grape Seed and Grape Seed Press Cake Grinding Procedures

Procedures used for grinding seeds and press cakes were modified during the course of this study. Originally grinding was accomplished by passing seeds and press cake through a Wiley mill (Thomas Scientific, Swedesboro, NJ) two times followed by two passes through a UDY mill (UD Corporation, Boulder, CO). During the original grinding procedure, fine sample particles adhering to surfaces inside the UDY mill were not collected. More particles accumulated inside the UDY mill during seed grinding compared to that of press cake grinding, which could be because of substantial differences in sample texture and/or higher oil content in seeds than that of press cake. The fine particles accumulated inside the mill were recovered and mixed with the remainder of the sample to avoid biasing of subsequent quantitative analysis between samples derived from seeds and from the press cake.

3.6 Sample Moisture Determination

Moisture content of ground samples were determined to allow expression of TPC and PA data on a dry weight basis. Ground samples (1 g) were accurately weighed into three tared aluminum containers. Samples were dried inside a forced draft oven at 75 ^{0}C until a constant weight was obtained. Samples were allowed to come to room temperature prior to weighing inside a desiccator to prevent moisture uptake during cooling for at least 20 min. Moisture content of the samples were determined by using the formula:

{(Initial sample weight - Dried sample weight)/ Initial sample weight}× 100

3.7 Grape seed and Grape Seed Press Cake Oil Determination

Since seed from grape varieties used for this study varied in oil content and oil yield during pressing of the seed was variety-dependent, oil content was determined to allow expression of seed and press cake TPC and PA determinations on a normalized oil-free basis. Triplicate ground samples (0.5 g) were weighed into 2 dram vials, a stir bar was added to each vial and 4 ml Diethyl ether was added. Securely capped vials were then stirred on a magnetic stirrer for 20 min followed by centrifugation at 3,000 g for 20 min using a Savant Speed Vac (Thermo Savent, Holbrook NY) centrifuge. Supernatants were transferred into new vials and evaporated for 20 min using the Speed Vac. The extraction/evaporation process was repeated 3 more times, for a total of 4 extractions. Particulates from the combined supernatants were mostly removed after the fourth

extraction by centrifuging the capped vials in the Speed Vac centrifuge for 20 min. Complete removal of remaining particles was achieved by filtration of the supernatant into a final tared 2 dram vial using a syringe filter with a 0.45 µm Nylon 66 membrane. Diethyl ether was then completely removed under vacuum in the Speed Vac for at least 3-4 hours. Samples were allowed to stand inside a fume hood overnight with lids loosely tightened and then the final weight was determined the next morning.

Calculation of oil content of the sample on a dry basis was done by using the formula given below:

[Oil wt / {starting wt - (starting wt × moisture percent)}]×100

3.8 Phenolic Extraction Procedures

3.8.1 *Extraction solvent pH adjustment***:**

Desired acidity of the extraction solvents was adjusted by adding hydrochloric acid or sodium hydroxide as required. A 209 R pH meter (Hanna instruments, Woonsocket, RI, US) was used to adjust the pH of the extraction solvents to 2 or 3. Solvents at the desired pH were stored at room temperature and used within 2 weeks.

3.8.2 *General extraction procedure***:**

We used 50% ethanol, 70% ethanol and 80% acetone, either without pH adjustment or after pH adjustment as described above, as extraction solvents for analytical evaluations. Water, either without pH adjustment or after pH adjustment as described above, was assessed as a phenolic partial extraction solvent. Triplicate ground samples (100 mg) were weighed into 2 dram vials and 4 ml of the appropriate extraction

solvent was added. Vials were securely capped and then incubated at various durations (1 h, 3 h or 6 h) with periodic mixing at 80 ^{0}C in a dry block heater. Two or three min after placement into the heating block, caps were retightened to prevent solvent evaporation during the incubation period. Vials were mixed every 15 min thereafter. After heating the samples for the desired amount of time, samples were cooled to room temperature and centrifuged in a Speed Vac at 3,000 g for 20 min. Samples were then filtered through Whatman #1 filter paper using a syringe filter. The volume of the filtrate was determined and the filtrate was then used for the quantification of total phenolic content (TPC) and proanthocyanidins (PA). The general extraction procedure for solid liquid extraction is shown in fig. 16.

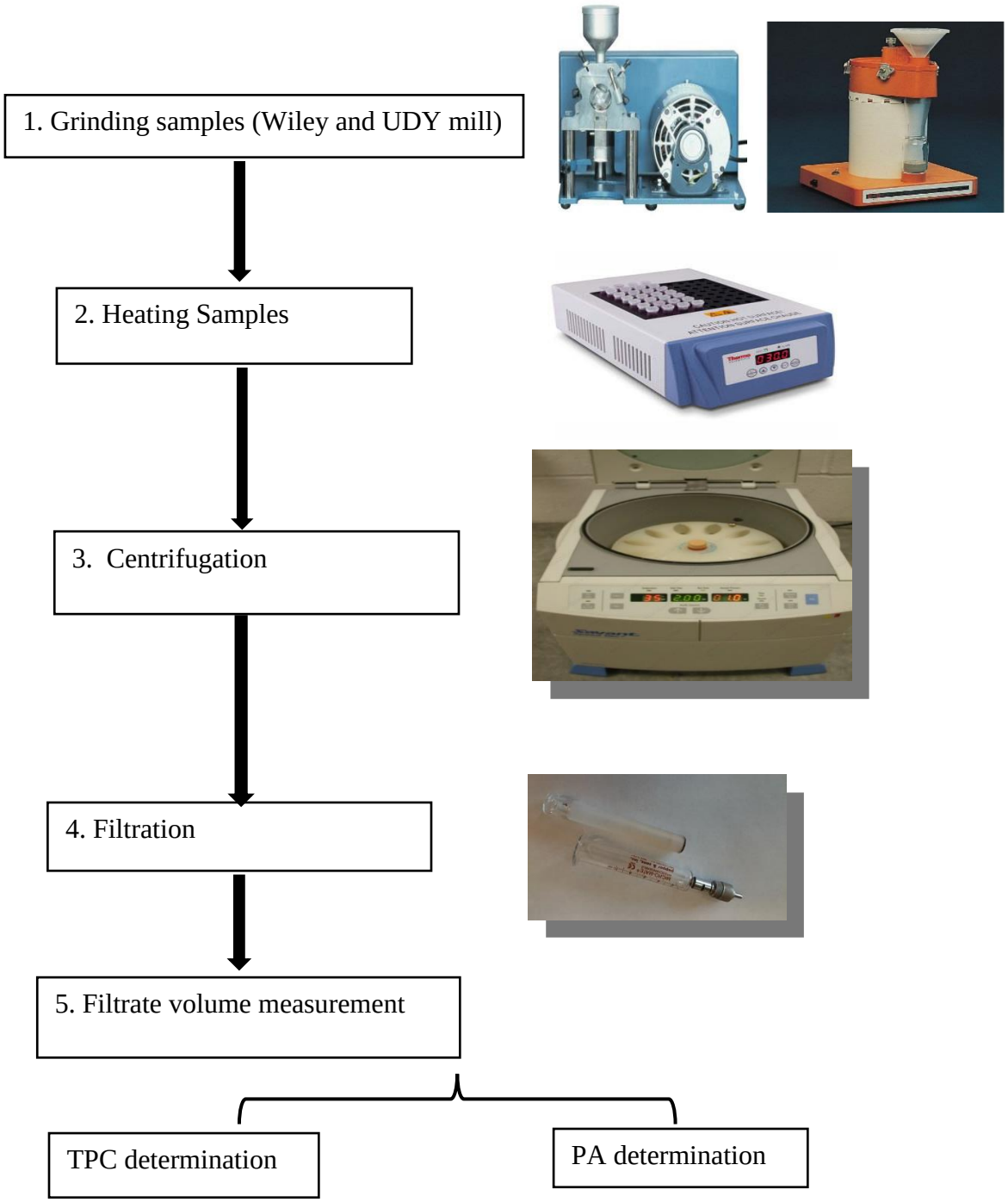

Fig. 16. General Solid Liquid extraction procedure for TPC and PA

3.9 Determination of Total Phenolic Content (TPC)

For the quantification of TPC, a Folin-Ciocalteau spectrophotometric micro method described by Waterhouse (1999) was followed using gallic acid as a standard. Total phenolic content was expressed in mg gallic acid equivalents per g sample on a moisture- and oil- free basis. Preparation of the various reagents required for the Folin-Cioclteau spectrophotometric micro method are briefly discussed below:

3.9.1 *Preparation of gallic acid stock solution:*

Gallic acid stock solution (0.5%) was required to prepare a standard curve, which was prepared by dissolving 0.5 g of gallic acid in 10 ml of ethanol (95%) and was brought to 100 ml volume with deionized water in a volumetric flask.

3.9.2 *Preparation of sodium carbonate solution:*

Sodium carbonate solution was prepared by dissolving 200 g of anhydrous sodium carbonate in 800 ml of deionized water. The solution was brought to a boil on a hot plate and after cooling, a few crystals of sodium carbonate were added to ensure that the solution is saturated. After 24 hours, the solution was filtered and the volume was brought to 1 liter with deionized water.

3.9.3 *Preparation of gallic acid standards:*

To prepare a calibration curve (Fig. 13), gallic acid stock solution was used. Gallic acid standard solutions of 50, 100, 150, 250 and 500 ppm were prepared by adding 0.1, 0.2, 0.3, 0.4, 0.5 and 1 ml of gallic acid stock solution into a 10 ml volumetric flasks

and bringing them to volume with deionized water. The gallic acid standard solutions were prepared fresh daily; the stock solution was prepared every day.

Total phenolic content of samples was determined after appropriate dilution with extraction solvent such that sample absorbance fell within the range of the gallic acid standard curve. Following dilution, the sample solutions were thoroughly mixed using a vortex mixer and 20 µl of sample or standard was added to 1.58 ml of water. A reagent blank was prepared in a 2 dram vial using 1.58 ml plus 20 µl of deionized water. Folin Ciocalteau reagent (100 µl) was then added to the samples, blank and calibration solutions and thoroughly mixed using a vortex mixer. Sodium carbonate solution (300 µl) was added to each vial within 8 min and mixed thoroughly using a vortex mixer. The vials were then loaded into a dry block heater maintained at 40 ^{0} C for 30 min. Vials were then allowed to cool to room temperature for about 20 min and absorbance readings of blank, sample and calibration solutions were taken at 765 nm using a Shimadzu UV 160 spectrophotometer (Shimadzu, Japan). The absorbance readings, corrected against the blank readings of calibration solutions, were further used to develop a standard curve for use in calculations to quantify the phenolic content in the sample.

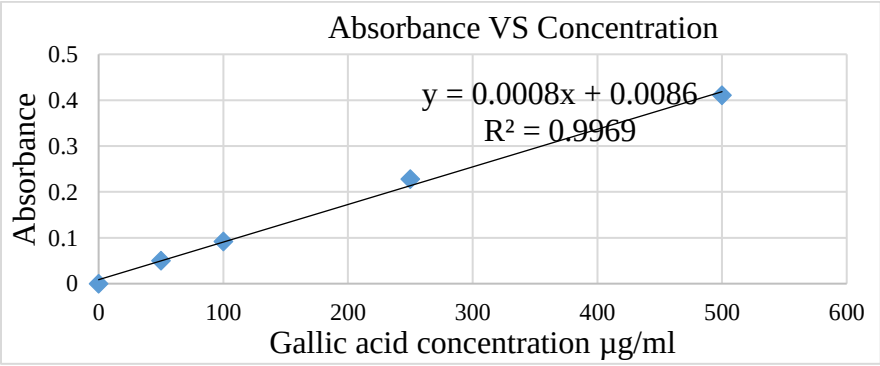

Fig 17. Calibration standard curve for Total phenolic content (TPC)

3.9.4 *Calculation of total phenolic content (TPC):*

Regression equation of the standard curve (Fig. 17) obtained after plotting the graph of calibration solution (absorbance Vs concentration) for each sample set was used to quantify TPC in mg/l. Total phenolic concentrations of sample extract was expressed in mg GAE/g dry sample using the formula:

(C×D×V) / (1000×S)

Where 'C' is the concentration of TPC in the sample extract in mg/l, 'D' is the dilution factor, 'V' is the extraction volume in milliliters and 'S' is the sample size in grams. Total phenolic content was finally expressed in terms of mg GAE/g dry, oil-free weight.

TPC mg GAE/g dry/oil-free weight was expressed using the formulae given below:

Firstly, TPC (mg GAE/g dry basis) = (TPC mg GAE/g)/ {(1- (moisture%/100)}

Secondly, TPC (mg GAE/g dry, oil-free) = TPC (mg GAE/g dry basis)/ {1- (oil%/100)}

3.10 Determination of Proanthocyanidins

Total proanthocyanidins was quantified using a vanillin colorometric method described by Prior et al. (2010) with proanthocyanidin B_2 as standard with some modifications. Volumes of sample and DMAC reagent added to the sample were scaled up keeping the ratio constant as described in the original procedure. Instead of adding 210 µl of DMAC solution to 70 µl of sample, 1200 µl of DMAC was added to 400 µl of

sample. Preparation of various reagents required for the vanillin colorometric method are briefly discussed below:

3.10.1 *Preparation of proanthocyanidin B_2 calibration standard*

Proanthocyanidin B_2 (2 mg) was accurately weighed into a 25 ml volumetric flask and brought to volume with 95% ethanol to obtain a standard stock solution of 40 µg/ml. The standard stock solution was stored in a freezer (stable for 6 months) and further used to prepare calibration solutions at different concentrations of 20 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml and 1.25 µg/ml daily by serial dilution.

3.10.2 *Preparation of Acidified ethanol*

Concentrated (36%) hydrochloric acid (12.5 ml) was added to 12.5 ml distilled water and 75 ml of 91% ethanol in a glass bottle and mixed properly. The solution was stored in the room temperature and could be used for a year.

3.10.3 *Preparation of Dilution solution*

Ethanol (95%) (80 ml) was added to 20 ml of deionized water and mixed properly. The solution was stored at room temperature.

3.10.4 *Preparation of DMAC reagent (0.1%)*

The appropriate quantity of a Dimethylacetamide (DMAC) powder was weighed out and added to an appropriate volume of acidified ethanol to prepare a 0.1% of the solution. The solution was mixed properly and the required volume of DMAC solution was made fresh daily.

Proanthocyanidin content of samples was determined after appropriate dilution with the dilution solution such that sample absorbance fell within the range of the proanthocyanidin standard curve. Following dilution, sample solutions were thoroughly mixed using a vortex mixer and 400 µl of sample or standard was added into 2 dram vials. Dilution solution (400 µl) was used as blank. DMAC solution (1.2 ml) was added to samples, blanks and calibration solutions. Absorbance readings of blanks, sample and calibration solutions were taken after incubation at room temperature for 15 to 30 min at 640 nm using a Shimadzu UV 160 Spectrophotometer (Shimadzu, Japan). Absorbance readings, corrected against the blank readings of calibration solutions were further used to develop a standard curve (Fig 18) for use in calculations to quantify the proanthocyanidin content in the sample.

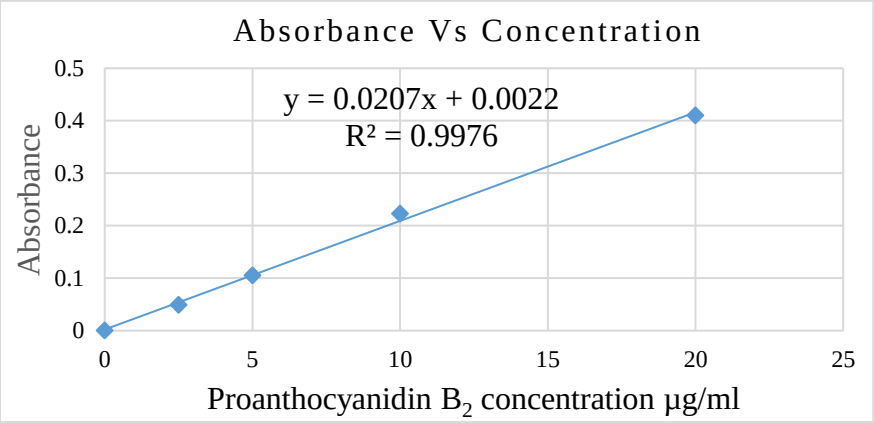

Fig 18. Calibration standard curve for Proanthocyanidin

Regression equation of the standard curve (Fig. 18) obtained after plotting the graph of calibration solution (absorbance Vs concentration) for each sample set was used to quantify PA in mg/l. Proanthocyanidin concentrations of sample extracts was expressed in mg proanthocyanidin B_2 equivalent /g using the formula:

(C×D×V) / (1000×S)

Where ‘C’ is the concentration of PA in the sample extract in mg/l, ‘D’ is the dilution factor, ‘V’ is the extraction volume in milliliters and ‘S’ is the sample size in grams. The proanthocyanidin concentration was finally expressed in terms of mg/g of proanthocyanidin B_2, dry, oil free weight.

PA mg proanthocyanidin B_2 equivalent/g dry, oil-free weight was expressed using the formula given below:

1. PA (mg proanthocyanidin B_2 equivalent / g dry basis) = (PA mg proanthocyanidin B_2 equivalent / g)/ {(1- (moisture% / 100)}
2. PA (mg proanthocyanidin B_2 equivalent / g dry, oil-free) = PA (mg proanthocyanidin B_2 equivalent /g dry basis) / {1- (oil%/100)}

3.11 Ultrasound-assisted Extraction (UAE)

Ultrasound-assisted extraction (UAE) was conducted with a Vibra-Cell™ Ultrasonic Liquid Processor Model VCX-750 (Sonics & Materials, Inc, CT, US). Major parts of ultrasonic liquid processor were ultrasonic electric generator, transducer and a solid probe (Fig 19). The processor was programmable to adjust variables such as amplitude, sonication time, energy and pulsing. The generator creates a signal (usually around 20 KHz) that powers a transducer. The transducer converts the electric signal to a mechanical vibration which is amplified by the sonicator and passed into the sample/solvent mixture through the probe immersed into the sample/solvent mixture. A 100 ml jacketed glass cell was used to maintain a desired temperature during sonication.

The desired extraction temperature (60 ^{0}C) during sonication was maintained with a water bath (Fisher Scientific, ISOTEMP 4100, PA, US).

3.11.1 *Extraction procedure*

Ground sample (1.5 g) was weighed into the 100 ml jacketed cell and 60 ml of extraction solvent was added. The water bath was set at the desired temperature prior to sonication such that during sonication a solution temperature of 60 ^{0}C was maintained. Water bath was set at the temperature lower than 60 ^{0}C based on the preliminary trials to subdue the continuous rise of sample/solvent temperature during sonication which if not controlled could reach the level that is degradative to phenolic compounds. The solid sonication probe (13 mm diameter) was immersed into the sample/solvent mixture and sonication was initiated. The sonicator was set at 80% amplitude, with continuous operation for the desired extraction time (0.5 min, 1 min, 2 min, 5 min, 10 min, 20 min, 40 min, 1 h, 2 h and 3 h). Solution temperature during extraction was monitored using a thermocouple immersed in the extraction solution.

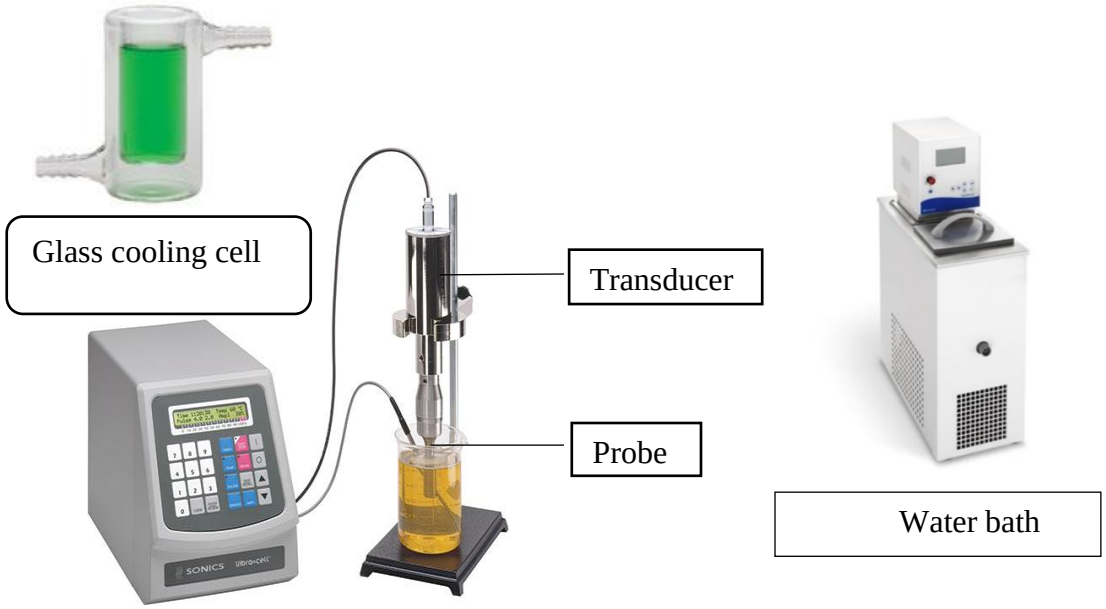

Fig. 19. Ultrasonic processor, cooling cell and water bath used in this study (From Sonics and Fisher scientific website)

After sonication, samples were transferred to centrifuge tubes and allowed to cool down for about 10 min before centrifuging for 20 min. After centrifugation, solution was filtered through Whatman #1 filter paper using a glass vacuum filter (Fig 20). Filtrate volume was recorded and TPC and PA was determined.

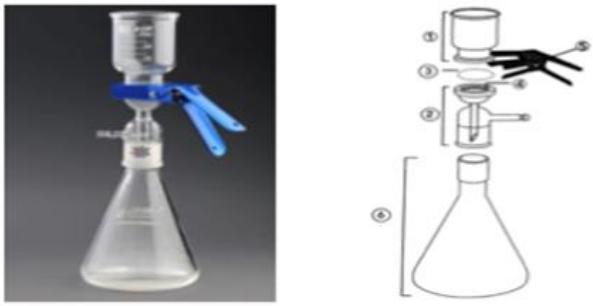

Fig 20. Glass vacuum filter

3.12 Experimental Design

3.12.1 Experimental Design for Extraction Using Organic Solvents

Completely randomized factorial design taking four factors with different levels was followed. The four factors considered were:

1) Grape variety: 'Merlot', 'Cabernet Sauvignon' and 'Sangiovese'
2) Extraction solvents: 80% acetone and ethanol (50%, and 70%).
3) pH levels of extraction solvents: pH 2 and pH 3
4) Extraction time: 1 h, 3 h and 6 h

3.12.2 Experimental Design for Extraction Using Water

Extraction of TPC and PA from grape seeds and press cake with water was conducted in a completely randomized factorial design with three factors at various levels. Factors considered were:

1) Grape variety: 'Merlot', 'Cabernet Sauvignon' and 'Sangiovese'
2) pH of water: pH 2, pH 3 and pH unadjusted (pH 5.4)
3) Extraction time: 1 h, 3 h and 6 h

3.12.3 Experimental Design for Ultrasound-assisted Extraction

Ultrasound-assisted extraction was conducted in a completely randomized factorial design was with two factors (treatment and time) at different levels given below:

1) Treatment: Ultrasound-assisted and control
2) Time: 5 min, 10 min, 20 min, 40 min, 60 min, 120 min and 180 min

This study was repeated again for different time duration (30 s, 1 min, 2 min, 5 min, 10 min, 20 min, 40 min and 60 min)

3.13 Data Analysis

All data were subjected to analysis of variance (ANOVA) using a mixed procedure in SAS software V 9.4 (SAS institute Cary NC). Treatment means were separated using Fisher's protected least significant difference (LSD) at $p = 0.05$. Triplicate measurements were done for all parameters. For each experiment, analysis of variance (ANOVA) was used to determine the fixed effects of factors and their interactions on the measured parameters (TPC and PA).

CHAPTER IV

RESULTS AND DISCUSSION

Although numerous protocols have been reported for phenolic and proanthocyanidin extraction from grape seed (Karvela et al., 2009b; Shi et al., 2003a; Spigno et al., 2007; Fontana et al., 2013), there does not appear to be a consensus on an ideal protocol. This study strived to understand the effect of several factors (solvent, solvent pH, extraction time and their interaction) on the phenolic compound extraction from grape seed and grape seed press cake following oil expression. Six hours of extraction was the longest time considered in our study because the majority of the previous research studies have suggested that optimum time for TPC and PA extraction is shorter than six hours, and longer extraction time might not be feasible economically in commercial extraction (Karvela et al., 2009b; Libran et al., 2013; Shi et al., 2003b). It is noteworthy that there are other factors such as extraction temperature, particle size and solid to solvent ratio which have also been reported to influence phenolic and proanthocyanidin extraction and that were fixed variables in this study. Extraction temperature was fixed in our work at 80 °C in accordance with preliminary results indicating that with extraction solvents of 80% acetone, 50% ethanol, 70% ethanol and water, the 80 °C extraction temperature resulted in the higher yields of total phenolic compounds (TPC) and proanthocyanidins (PA) than 60 °C or 70 °C (data not shown). Sample grinding procedure also had an impact on TPC and PA yield from grape seed and

grape seed press cake following oil expression. In preliminary work, grinding twice with a Wiley mill followed by two passes through a UDY mill were necessary to achieve highest yields; in fact, small particle sizes tended to accumulate inside the UDY mill, which will be shown to have a positive impact on total TPC and PA when recovered and included as part of our sample, especially when water was used as partial extraction solvent. We settled on a 40:1 solvent to solids ratio for these extraction studies due to long-standing protocols followed in our lab, which agree with previous recommendations of solvent to solids ratio for grape seed extraction of 40:1 or 50:1 (Bucić-Kojić et al., 2007; Sant'anna et al., 2012).

This work was conducted using seed and press cake after seed oil expression from locally-grown red wine grapes 'Merlot', 'Cabernet Sauvignon' and 'Sangiovese' obtained after vinification from an Oklahoma winery. Phenolic content of grape seed has been shown to vary depending on the geographic region in which grapes are grown (Bozan et al., 2008; Pantelic et al., 2016; Shi et al., 2003b); our work not only evaluates extraction conditions to optimize TPC and PA yield from grape seed but also documents concentrations of these products in seed from our growing region. This contributes to an overall goal of decreasing waste generation by local wineries by utilizing valuable components to produce marketable products.

Extraction conditions were evaluated in a methodical fashion. Firstly, the effect of solvent pH (for each extraction solvent at each level of extraction time) on TPC and PA yield was assessed and the best solvent pH was selected for further comparisons. Secondly, the effect of extraction time on TPC and PA yield was evaluated for all

solvents at one pH level. Thirdly, comparison of extraction solvents was done at the selected time and pH level.

4.1 Optimizing TPC and PA Extraction Using Organic Solvents (80% acetone, 50% ethanol and 70% ethanol)

We evaluated the effect of different factors and their interactions on TPC and PA extraction yield from grape seed and press cake to optimize the extraction conditions. The influence of extraction factors on TPC and PA are discussed below under different headings and subheadings.

4.1.1 Effect of Solvent pH on TPC and PA Extraction from Grape Seeds and Press Cake

The effect of solvent pH of 2 or 3 on TPC and PA from grape seeds and grape seed press cake of each grape variety, 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' was found highly significant. However, the interaction of solvent pH with other extraction variables (time, solvent and variety) was variable and differed depending on whether yield of TPC or of PA was being assessed. Effect of solvent pH on TPC and PA extraction from grape seeds and grape seed press cake of three different Oklahoma grown grape varieties is discussed for TPC and PA separately below:

4.1.1a *Effect of solvent pH on TPC extraction*

Effect of solvent pH on TPC extraction is presented in Table 1. All combinations exhibited increasing TPC yield with longer extraction time. Among all solvents, 50% ethanol and 70% ethanol adjusted at pH 2 yielded significantly higher TPC than these solvents adjusted to pH 3 from seed and press cake of each grape variety for each

extraction time. However, the effect of pH on 80% acetone in terms of TPC extraction varied for grape varieties. TPC was significantly higher for 80% acetone, pH 2 from seed and press cake of ‘Cabernet Sauvignon’ at each level of extraction time (1 h, 3 h and 6 h). For ‘Merlot’ seed and press cake, pH 2 adjusted 80% acetone gave significantly higher TPC only at 3 h and 6 h of extraction. No significant effect of pH with 80% acetone was found for TPC from ‘Sangiovese’ seed and press cake.

Table 1. Effect of pH on TPC (mg GAE/g dry, oil-free sample) from 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' seed and press cake using different extraction solvents (80% acetone, 50% ethanol and 70% ethanol) at different levels of extraction time (1 h, 3 h and 6 h) (n = 3).

Variety	Time	pH	80% Acetone				50% Ethanol				70% Ethanol			
			Seed		Press Cake		Seed		Press Cake		Seed		Press Cake	
			TPC	Sign [z]	TPC	Sign	TPC	S	TPC	Sign	TPC	Sign	TPC	Sign
Cabernet Sauvignon	1	2	57.34	**[y]	62.47	*	53.78	***	80.81	***	62.75	***	89.60	***
		3	47.79		58.30		37.05		54.72		41.85		51.22	
	3	2	66.50	***	74.83	**	69.20	***	85.92	***	72.72	***	104.78	***
		3	54.78		69.49		51.94		64.85		55.99		63.27	
	6	2	88.66	***	87.83	**	72.01	***	98.79	***	88.74	***	102.75	***
		3	76.31		80.92		56.34		74.95		57.70		66.03	
Merlot	1	2	67.84	NS	93.86	**	71.18	***	100.29	***	74.83	***	102.02	***
		3	70.32		88.03		54.58		71.33		58.56		70.62	
	3	2	75.41	**	101.84	*	78.74	***	108.68	***	86.51	***	114.89	***
		3	67.18		97.82		67.50		82.96		69.63		77.40	
	6	2	87.52	**	103.60	NS [x]	88.75	***	108.63	***	93.51	***	120.88	***
		3	80.12		100.59		76.92		91.11		73.05		87.67	
Sangiovese	1	2	61.39	NS	64.70	NS	59.60	***	73.11	***	79.02	***	68.77	***
		3	62.76		66.77		47.81		56.18		48.07		50.89	
	3	2	63.75	NS	73.94	NS	71.12	***	82.20	***	78.61	***	77.14	***
		3	64.18		71.29		57.27		63.20		55.83		55.89	
	6	2	72.22	NS	74.22	NS	75.08	***	87.28	***	82.80	***	83.56	***
		3	70.07		73.36		58.44		65.76		59.76		61.26	

[z] Sign denotes significance.

[y] * Significance at $\alpha = 0.05$, **. Significance at $\alpha = 0.01$, *** Significance at $\alpha = 0.001$.

[x] NS indicates no significant difference.

4.1.1b *Effect of solvent pH on PA extraction*

The effect of solvent pH on PA extraction is presented in Table 2. Unlike results found for TPC in which TPC yields increased for all combinations with increase in extraction time, PA yields appeared to increase from 1 h to 3 h of extraction but did not increase with an additional 3 h of time to 6 h of extraction. In most of the cases, there was no significant influence of pH on PA yield using 80% acetone as extraction solvent.

PA yield from grape seeds and press cake with 50% ethanol was found to be significantly higher for pH 2 compared to pH 3 in most of the cases except for 'Cabernet Sauvignon' and 'Sangiovese' at 3h for seeds and 'Merlot' at 6h and 'Sangiovese' at 3h and 6 h for press cake. Effect of pH on PA from grape seed with 70% ethanol was less clear, especially in regards to PA yield from seed. While PA yields from press cake appeared to be favored by pH 2 versus pH 3 during 1 h and 3 h of extraction for all varieties, seed PA yields showed this trend for only one variety ('Cabernet Sauvignon'). In all cases, however, when pH did influence PA extraction yield, pH 2 was higher than pH 3.

Table 2. Effect of pH on PA (mg proanthocyanidin B2 equivalent/g dry, oil-free sample) extraction from 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' seed and press cake using different extraction solvents (80% acetone, 50% ethanol and 70% ethanol) at different levels of extraction time (1 h, 3 h and 6 h) (n = 3).

Variety	Time	pH	80% Acetone				50% Ethanol				70% Ethanol			
			Seed		Press Cake		Seed		Press Cake		Seed		Press Cake	
			PA	Sign [z]	PA	Sign	PA	Sign	PA	Sign	PA	Sign	PA	Sign
Cabernet Sauvignon	1	2	35.98	*** [y]	31.61	NS	31.74	**	39.60	***	35.76	**	46.09	***
		3	28.50		30.36		26.70		30.38		29.98		35.30	
	3	2	41.56	NS [x]	37.83	NS	40.35	NS	44.67	***	48.17	**	48.74	***
		3	40.46		38.26		40.51		39.47		42.60		39.57	
	6	2	42.53	NS	40.70	NS	38.41	*	44.85	***	44.17	NS	45.84	*
		3	42.18		41.40		42.80		38.60		46.56		43.00	
Merlot	1	2	51.54	NS	55.02	NS	45.40	*	58.98	***	47.04	NS	60.89	***
		3	50.45		58.10		41.79		46.90		44.63		46.94	
	3	2	62.79	NS	56.62	NS	53.14	*	60.94	**	57.76	NS	58.28	***
		3	58.39		56.72		57.24		56.20		56.63		50.44	
	6	2	68.22	NS	55.62	NS	57.66	**	57.98	NS	52.25	***	58.49	NS
		3	65.85		60.05		63.99		59.95		62.90		59.58	
Sangiovese	1	2	48.60	NS	45.21	NS	42.77	**	43.92	**	48.23	**	47.49	***
		3	46.24		45.67		36.72		40.38		43.10		40.75	
	3	2	57.14	NS	51.26	NS	52.05	NS	47.95	NS	56.54	NS	54.16	***
		3	55.74		53.21		48.97		47.13		53.75		49.18	
	6	2	62.36	**	50.68	*	52.14	*	48.10	NS	54.66	NS	52.13	NS
		3	57.28		53.16		56.45		50.22		57.95		51.35	

[z] Sign denotes significance.

[y] * Significance at $\alpha = 0.05$, **. Significance at $\alpha = 0.01$, *** Significance at $\alpha = 0.001$.

[x] NS indicates no significant difference.

Although our results did not show a universal positive influence of pH 2 solvent adjustment over pH 3 for TPC and PA extraction yield, when there was an influence pH 2 exhibited highest yield. Other research studies have suggested that lower extraction solvent pH was better for TPC and PA yield from grape seed (Libran et al., 2013; Karvela et al., 2009), but no studies have compared the narrow change from pH 2 to pH 3. Increased recovery of TPC and PA with decreasing solvent pH from 3 to 2 might be due to higher dissociation of the most acid phenolic –OH groups which enhanced polyphenol solubility by increasing polyphenols' polarity (Mylonaki, 2008). Libran et al. (2013) found significantly higher TPC yield for pH 2 adjusted ethanol (50% and above) compared to pH 5.3 and higher pH. Using response surface methodology, Karvela et al. (2009) found that the lower pH (2 - 3.26) of ethanol at various concentrations were optimum for TPC and PA extraction.

This study demonstrated that even a small change in the extraction solvent pH could have a remarkable impact on TPC and PA yield, although the influence was not equivalent for the two phenolic components. This could be due to the combined effect of the variation in the individual phenolic profile of grape varieties, different degree of pH sensitivity of individual phenolic compound, different polarities of the extraction solvents and different effect of acidity in solvents with different polarities (Lin and Giusti, 2005). In all cases where pH was shown to influence phenolic yield, pH 2 was favored over pH 3; pH 2 was chosen to go forward with our evaluations of extraction time and extraction solvents.

4.1.2 Effect of Extraction Time on TPC and PA Extraction from Grape Seeds and Press Cake

Effect of time on TPC and PA extraction was evaluated for different extraction solvents (80% acetone, 50% ethanol and 70% ethanol) adjusted at pH 2. Time (1 h, 3 h and 6 h) had a significant effect on the extraction of TPC and PA from grape seeds and grape seed press cake. Effect of extraction time on TPC and PA is discussed separately below:

4.1.2a *Effect of extraction time on TPC*

Effect of extraction time on TPC is presented in Table 3. Total phenolic content from seed and press cake from each grape variety for each solvent was found significantly higher for extraction time above 1 h except from 'Sangiovese' seed extracted with 70% ethanol (pH 2). Depending on the sample material (either seed or press cake) and grape variety, TPC at 6 h was either significantly higher than 3 h or there was no significant difference in TPC yield between 3 h and 6 h of extraction. Samples exhibiting no significant difference between 3 h and 6 h of extraction for TPC included 'Merlot' and 'Sangiovese' press cake with 80% acetone, 'Cabernet Sauvignon' and 'Sangiovese' seed with 50% ethanol and 'Cabernet Sauvignon' press cake with 70% ethanol.

Table 3. Effect of extraction time on TPC (mg GAE/g dry, oil free sample) extraction from 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' seed and press cake using different extraction solvents (80% acetone, 50% ethanol and 70% ethanol) adjusted at pH 2 (n = 3).

Variety	Time	80% Acetone (pH 2)				50% Ethanol (pH 2)				70% Ethanol (pH 2)			
		Seed		Press Cake		Seed		Press Cake		Seed		Press Cake	
		TPC	Sign [z]	TPC	Sign	TPC	Sign	TPC	Sign	TPC	Sign	TPC	Sign
Cabernet Sauvignon	1	57.3 c	*** [y]	62.5 c	***	53.8 b	***	80.8 c	***	62.7 c	***	89.6 b	***
	3	66.5 b		74.8 b		69.2 a		85.9 b		72.7 b		104.8 a	
	6	88.7 a		87.8 a		72.0 a		98.8 a		88.7 a		102.8 a	
Merlot	1	67.8 c	***	93.9 b	***	71.2 c	***	100.3 b	***	74.8 c	***	102.0 c	***
	3	75.4 b		101.8 a		78.7 b		108.7 a		86.5 b		114.9 b	
	6	87.5 a		103.6 a		88.8 a		108.6 a		93.5 a		120.9 a	
Sangiovese	1	61.4 b	***	64.7 b	***	59.6 b	***	73.1 c	***	79.0 a	NS [x]	68.8 c	***
	3	63.8 b		73.9 a		71.1 a		82.2 b		78.6 a		77.1 b	
	6	72.2 a		74.2 a		75.1 a		87.3 a		82.8 a		83.6 a	

[z] Sign denotes significance.

[y] * Significance at $\alpha = 0.05$, **. Significance at $\alpha = 0.01$, *** Significance at $\alpha = 0.001$

[x] NS indicates no significant difference.

[w] Common letter within each column indicates no significant difference ($p < 0.05$).

4.1.2b *Effect of extraction time on PA*

The effect of extraction time on PA yield is shown in Table 4. Like results described for TPC yield, PA from grape seed and press cake of each variety with each solvent, with the exception of 'Merlot' press cake, was also significantly higher for extraction time above 1 h. For all solvents (80% acetone, 50% ethanol and 70% ethanol), time had no significant effect on PA from 'Merlot' press cake. In the majority of the cases, there was no significant difference in PA yield between 3 h and 6 h. Unlike results found for TPC in which TPC yields increased for all combinations with increase in extraction time, PA yields appeared to increase from 1 h to 3 h of extraction but did not increase in most cases with an additional 3 h of time to 6 h of extraction.

Table 4. Effect of extraction time on PA (mg proanthocyanidin B2 equivalent/g dry, oil-free sample) extraction from 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' seed and press cake using different extraction solvents (80% acetone, 50% ethanol and 70% ethanol) adjusted at pH 2 (n = 3).

Variety	Time	80% Acetone (pH 2)				50% Ethanol (pH 2)				70% Ethanol (pH 2)			
		Seed		Press Cake		Seed		Press Cake		Seed		Press Cake	
		PA	Sign [z]	PA	Sign	PA	Sign	PA	Sign	PA	Sign	PA	Sign
Cabernet Sauvignon	1	36.0 b	*** [y]	31.6 c	***	31.7 b	***	39.6 b	***	35.8 c	***	46.1 b	*
	3	41.6 a		37.8 b		40.4 a [w]		44.7 a		48.2 a		48.7 a	
	6	42.5 a		40.7 a		38.4 a		44.8 a		44.2 b		45.8 b	
Merlot	1	51.5 c	***	55.0 a	NS [x]	45.4 c	***	59.0 a	NS	47.0 c	***	60.9 a	NS
	3	62.8 b		56.6 a		53.1 b		60.9 a		57.8 a		58.3 a	
	6	68.2 a		55.9 a		57.7 a		58.0 a		52.2 b		58.5 a	
Sangiovese	1	48.6 c	***	45.2 b	***	42.8 b	***	43.9 b	***	48.2 b	***	47.5 b	***
	3	57.1 b		51.3 a		52.1 a		48.0 a		56.5 a		54.2 a	
	6	62.4 a		50.7 a		52.1 a		48.1 a		54.7 a		52.1 a	

[z] Sign denotes significance.

[y] * Significance at α = 0.05, **. Significance at α = 0.01, *** Significance at α = 0.001

[x] NS indicates no significant difference.

[w] Common letter within each column indicates no significant difference ($p < 0.05$).

These results indicated that the 6 h of extraction was probably needed for maximum recovery of TPC from grape seeds and press cake. However, in some cases, depending on the grape variety, solvent and sample material (seed or press cake) TPC yield might not increase significantly with extraction time longer than 3 h. Shi et al. (2003 b) found that duplicate extractions of 1.5 h each were necessary for maximum yield of TPC from grape seed press cake at 65 ^{0}C using 50% ethanol. This finding is in agreement with our results that the extraction of TPC from 'Merlot' and 'Sangiovese' seed press cake was optimum at 3 h using 50% ethanol. Some studies have suggested that an extraction time above 6 h (up to 24 h) may be needed to get maximum recovery of TPC from grape pomace (Lapornik et al., 2005; Spigno et al., 2007). As reported by Karvela et al. (2009a), the results suggested that the optimum time for TPC extraction from the same plant tissue (grape seeds and grape seed press cake in our case) using the same solvent could be different for different varieties. Karvela et al. (2009a) found different optimum extraction times (1 h, 3 h and 5 h) for maximum TPC yield from grape stems of different grape varieties using aqueous acidified ethanol.

Results also suggested that the extraction of PA might not significantly increase with extraction time above 3 h. The results suggested that PA could be optimally extracted within the shorter time of 3 h compared to that of TPC which appeared to require 6 h of extraction for maximum yield. This result is in agreement with Karvela et al. (2009b) who reported that the shorter extraction time of 1 h was enough for PA extraction compared to that of TPC extraction which required 5 h for optimum yield from 'Moschofilero' grape seeds using acidified ethanol (40% for TPC and 52.1% for PA).

However, Huh et al. (2004) reported that an 8 h extraction duration was optimal for maximum oligomeric proanthocyanidin yield from wild grape seeds using 70% ethanol.

Our results suggested that 3 h of extraction was sufficient to achieve maximum extraction in many cases for PA and in some cases for TPC, but that 6 h extraction duration was required in other cases. The 6 h extraction duration, with solvent pH adjusted to 2, was chosen to continue our evaluation of extraction solvent on TPC and PA yield from grape seed and grape seed press cake.

4.1.3 Solvent Comparison for TPC and PA Extraction from Grape Seeds and Press Cake

Different extraction solvents (80% acetone, 50% ethanol and 70% ethanol) adjusted at pH 2 were compared after 6 h extraction durations in terms of TPC and PA extraction yield from grape seed and press cake of 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' grape varieties. Our results are discussed for TPC and PA separately below:

4.1.3a *Effect of solvent on TPC*

Results for the solvent comparison in terms of TPC is shown in the table 5. Except for 'Sangiovese' press cake, TPC yield from seeds and press cake in each variety was either significantly higher for 70% ethanol or equivalent compared to other solvents. Extraction solvent did not significantly impact TPC yield from 'Merlot' seeds. In 'Cabernet Sauvignon' seed, there was no significant difference in TPC between 80% acetone and 70% ethanol, while 50% ethanol gave the lowest TPC yield.

Table 5. Effect of different solvents (80% acetone, 50% ethanol and 70% ethanol) adjusted at pH 2 on TPC (mg GAE/g dry, oil-free sample) from 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' seeds and press cake extracted for 6 hours (n = 3).

Variety	Solvent (pH 2)	Seed		Press Cake	
		TPC	Sign [z]	TPC	Sign
Cabernet Sauvignon	Acetone 80% Ethanol 50% Ethanol 70%	88.7 a [w] 72.0 b 88.7 a	*** [y]	87.8 c 98.9 b 102.8 a	***
Merlot	Acetone 80% Ethanol 50% Ethanol 70%	87.5 a 88.8 a 93.5 a	NS [x]	103.6 c 108.6 b 120.9 a	**
Sangiovese	Acetone 80% Ethanol 50% Ethanol 70%	72.2 b 75.1 b 82.8 a	**	74.2 c 87.3 a 83.6 b	**

[z] Sign denotes significance.

[y] * Significance at $\alpha = 0.05$, **. Significance at $\alpha = 0.01$, *** Significance at $\alpha = 0.001$.

[x] NS indicates no significant difference.

[w] Common letter within each column indicates no significant difference ($p < 0.05$).

4.1.3b *Effect of solvent on PA*

Results for the solvent comparison in terms of PA is shown in the table 6. Except for 'Cabernet Sauvignon' press cake, PA from seeds and press cake of each variety was either significantly higher for 80% acetone or equivalent to one or more solvents.

Extraction solvent was found to have no significant effect on PA yield from ‘Merlot’ press cake.

Table 6. Effect of different solvents (80% acetone, 50% ethanol and 70% ethanol) adjusted at pH 2 on PA (mg proanthocyanidin B_2 equivalent/g dry, oil-free sample) from ‘Cabernet Sauvignon’, ‘Merlot’ and ‘Sangiovese’ seeds and press cake extracted for 6 hours (n = 3).

Variety	Solvent	Seed		Press Cake	
		PA	Sign [z]	PA	Sign
Cabernet Sauvignon	Acetone 80% Ethanol 50% Ethanol 70%	42.5 a 38.4 b 44.2 a	**[y]	40.7 b 44.9 a 45.8 a	***
Merlot	Acetone 80% Ethanol 50% Ethanol 70%	68.2 a 57.7 b 52.3 c	***	55.9 a [w] 58.0 a 58.5 a	NS [x]
Sangiovese	Acetone 80% Ethanol 50% Ethanol 70%	62.4 a 52.1 b 54.7 b	***	50.7 a 48.1 b 52.1 a	**

[z] Sign denotes significance.

[y] * Significance at $\alpha = 0.05$, **. Significance at $\alpha = 0.01$, *** Significance at $\alpha = 0.001$.

[x] NS indicates no significant difference.

[w] Common letter within each column indicates no significant difference ($p < 0.05$).

Our findings indicated that the optimal solvent for TPC from both seed and press cake appeared to be 70% ethanol whereas extraction of PA is mostly favored by 80% acetone. These results are in accordance with some other investigations which reported that ethanol/water mixtures were found relatively better than acetone/water mixtures for

TPC extraction (Fontana et al., 2013) and acetone/water mixture was better than the ethanol/water mixture for PA extraction (Yilmaz and Toledo, 2006). Extraction potential of different solvents varied with the type of phenolic compounds (TPC and PA) which could be due to the difference in solvent polarity and the differential affinity of specific phenolic compounds towards extraction solvent. In order to adequately document TPC and PA yield from grape seed and grape seed press cake for our local varieties, 70% ethanol was chosen as extraction solvent to assess TPC content and 80% acetone was chosen to assess PA content, with both solvents adjusted to pH 2 and for an extraction duration of 6 h.

4.1.4 Varietal Comparison for TPC and PA from Seeds and Press Cake

Total phenolic content and PA from seed and press cake of three different Oklahoma grown varieties, 'Merlot', 'Cabernet Sauvignon' and 'Sangiovese' are shown in the Table 7. Total phenolic content from grape seeds were in the following order from high to low concentration: 'Merlot' (93.5 mg GAE/g dry, oil-free seed) > 'Cabernet Sauvignon' (88.7 mg GAE/g dry, oil-free seed) > Sangiovese (82.8 mg GAE/g dry, oil-free seed). Proanthocyanidin from grape seeds were in the following order from high to low concentration: 'Merlot' (65.9 mg proanthocyanidin B_2 equivalent/g dry, oil-free sample) > 'Sangiovese' (57.3 mg proanthocyanidin B_2 equivalent/g dry, oil-free sample) > 'Cabernet Sauvignon' (42.2 mg proanthocyanidin B_2 equivalent/g dry, oil-free sample). Total phenolic content from press cake were in the following order from high to low concentration: 'Merlot' (120.9 mg GAE/g dry, oil-free sample) > 'Sangiovese' (102.8 mg GAE/g dry, oil-free sample) > 'Cabernet Sauvignon' (83.6 mg GAE/g dry, oil-free sample). Proanthocyanidin from press cake were in the following order from high to low

concentration: ‘Merlot’ (55.9 mg proanthocyanidin B_2 equivalent/g dry, oil-free sample) > ‘Sangiovese’ (50.7 mg proanthocyanidin B_2 equivalent/g dry, oil-free sample) > ‘Cabernet Sauvignon’ (40.7 mg proanthocyanidin B_2 equivalent/g dry, oil-free sample). This result documented that the TPC and PA concentrations were different for seed and press cake of different grape varieties grown and processed for wine in Oklahoma.

Table 7. TPC and PA from seed and press cake of ‘Cabernet Sauvignon’, ‘Merlot’ and ‘Sangiovese’ extracted for 6 hours at pH 2. 70% ethanol and 80% acetone were used for TPC and PA extraction, respectively (n = 3).

Grape Variety	TPC (mg GAE/g dry, oil-free sample)		PA (mg proanthocyanidin B_2 equivalent/g dry, oil-free sample)	
	Seed	Press Cake	Seed	Press Cake
Cabernet Sauvignon	88.7 a	102.8 b	42.2 c	40.7 c
Merlot	93.5 a [z]	120.9 a	65.9 a	55.9 a
Sangiovese	82.8 b	83.6 c	57.3 b	50.7 b

[z] Common letter within each column indicates no significant difference ($p < 0.05$).

These results are in accordance with a previous investigation where Bozan et al. (2008) reported no significant difference in the TPC yield between ‘Merlot’ and ‘Cabernet Sauvignon’ seeds grown in Turkey. However, the same study found proanthocyanidin significantly higher for ‘Cabernet Sauvignon’ compared to that of ‘Merlot’ whereas we document considerably lower PA in ‘Cabernet Sauvignon’

compared to ‘Merlot’. Pantelic et al. (2016) found higher TPC in ‘Merlot’ followed by ‘Cabernet Sauvignon’ and ‘Sangiovese’ respectively from Siberian grape seeds.

Since TPC and PA content in grape seed has been expressed as a percentage of seed weight in previous studies (Markis et al., 2007; Shi et al., 2003 a) rather than as a concentration in data shown in Table 7, our results were converted on a dry, oil-free basis in grape seed and press cake of different grape varieties and present the data in Table 8. TPC in grape seeds ranged from 8.3% to 9.4% of seed weight on dry, oil free basis. This result is slightly higher than what other studies have reported in general, in part due to our adjustment of results to account for oil content. According to Shi et al. (2003b), TPC accounts for 5%-8% grape seeds weight on a dry (but not oil-free) basis. On a dry (but not oil-free) basis TPC from grape seeds in our study ranged from 7.3% to 8.4% which is in agreement with Shi et al. (2003b). Proanthocyanidins in grape seeds from our study ranged from 4.3% to 6.8% on a dry, oil-free basis. In our study, proanthocyanidins represented 48.9% to 74.7% of total polyphenols in the grape seed (Table 8). This finding is in accordance with Mylonaki et al. (2008) who reported that the procyanidins represents 50% - 70% of total polyphenols in grape seed. Total phenolic content in press cake ranged from 8.4% to 12.1% on a dry, oil-free weight basis. Our TPC from press cake was double than what Shi et al. (2003a) found. The lower TPC content in the study done by Shi et al. (2003) could be because they extracted TPC using 50% ethanol for 1.5 h at 65 ^{0}C and we extracted TPC from cake using 70% ethanol for 6 h using at 80 ^{0}C. Higher temperature, longer extraction time, higher ethanol concentration could have caused increased TPC recovery from press cake in our study. Proanthocyanidins in press

cake ranged from 4.1% to 5.6% on dry, oil-free weight basis. Proanthocyanidins represented 39.8% to 60.7% of total polyphenols in the press cake.

Table 8. Percentage TPC and PA from 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' seeds and press cake extracted for 6 h with 70% ethanol (pH 2) and 80% acetone (pH 2) (n = 3).

Variety	Sample	TPC [z]	PA [y]	Percentage of PA in TPC
Cabernet Sauvignon	Seed	8.8	4.3	48.9
Merlot		9.4	6.8	72.3
Sangiovese		8.3	6.2	74.7
Cabernet Sauvignon	Cake	10.3	4.1	39.8
Merlot		12.1	5.6	46.3
Sangiovese		8.4	5.1	60.7

[z] TPC in g GAE/100 g dry, oil-free sample. TPC was extracted for 6h with 70% ethanol at pH 2.

[y] PA value in g proanthocyanidin B2 equivalent/100 g dry, oil-free sample. PA was extracted for 6 h with 70% ethanol (pH 2).

4.2 Effect of Grinding Procedure on TPC and PA

Our finding that TPC content of grape seed press cake was higher than that of grape seeds appeared curious, since some TPC should have been lost due to heat degradation and fines loss within the expressed oil pressing (Maier et al., 2009). Our procedures were further investigated to evaluate whether this apparent anomaly for TPC results was true or was imposed somehow by our procedures. Grape seed press cake has been reported to contain less total phenolic compounds compared to that of seeds which could be due to the thermal degradation of phenolic compounds during seed oil pressing (Maier et al., 2009).

During our re-evaluation of our procedures, it was found that some differences could be noted in residual sample distribution during our grinding procedure. In the original grinding procedure, samples were passed through a Wiley mill twice followed by two passes through a UDY mill. We examined the grinding chamber of the UDY mill and found accumulation of fine particles inside the mill, especially when seeds were being ground as opposed to press cake (Fig 21). The quantity of fine particles trapped around the black rubber ring underneath the mill cover and along the upper portion of the grinding ring where the black rubber sealed during grinding was notably higher for grape seed (b and d) compared to that of grape seed press cake (a and c). The substantial difference in the texture between grape seed and press cake and higher oil content in grape seeds could have caused more particles to accumulate inside the UDY mill while grinding seeds.

Fig. 21. Different parts of UDY mill; Mill cover after grinding press cake (b) and grape seed (a), grinding ring and chamber after grinding press cake (c) and grape seed (d)

Trials were conducted with ‘Sangiovese’ grape seed and press cake and extracted TPC and PA using 50% ethanol adjusted at pH 2 for 3 h of extraction. Inclusion of the fines trapped in the UDY mill with the rest of the sample which passed through the mill resulted in higher TPC (Fig. 22) and PA (Fig. 23) for seeds compared to that of press cake and was termed our modified grinding procedure. We postulate that inclusion of the finer particles in the sample using modified grinding procedure gave the higher TPC and PA yields from grape seeds versus press cake because the particular fine particles omitted from the ground sample may have originated from the outer soft coat of seed which are particularly rich in polyphenols, especially procyanidins (Thorngate and Singleton, 1994). Both TPC and PA from seed increased significantly with our modified grinding procedure compared to that of original grinding procedure; in the case of press cake, grinding procedure did not significantly influence PA concentration (Fig 25), while TPC concentration was significantly higher for the original grinding procedure (Fig 24).

Passing sample material through wiley mill twice followed by two passes through UDY mill and collecting the fine particles adhered inside the UDY mill appeared to be the better grinding procedure. Our modified grinding procedure was used for further extraction studies with water and ultrasound.

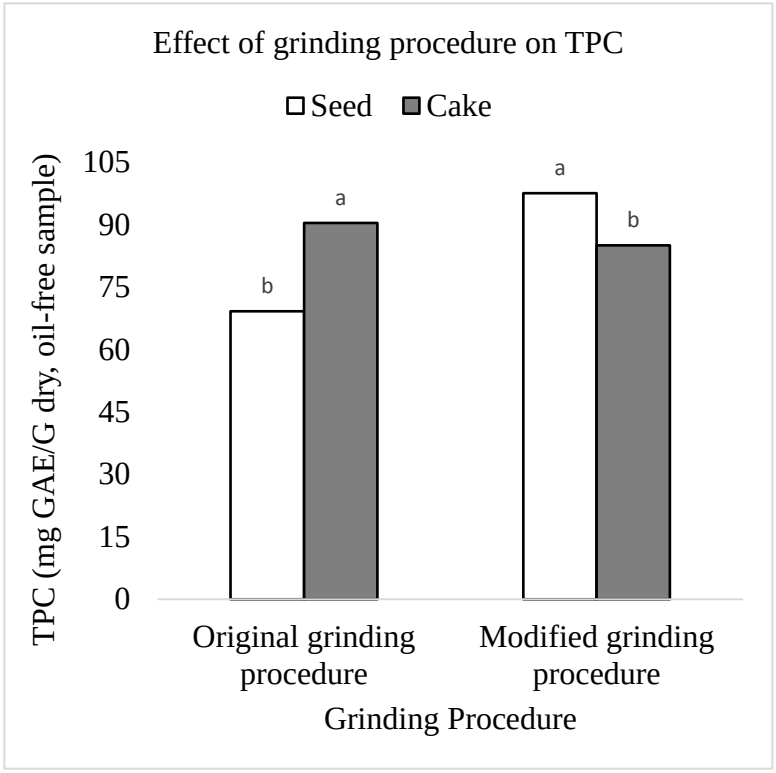

Fig 22. Comparison of grape seed and press cake in terms of TPC with different grinding procedures. TPC extracted from seeds and press cake of 'Cabernet Sauvignon' using 50% ethanol at pH 2 for 3 h of extraction (n = 3). Bar within grinding procedure with same group with different letters were significantly different ($p < 0.05$)

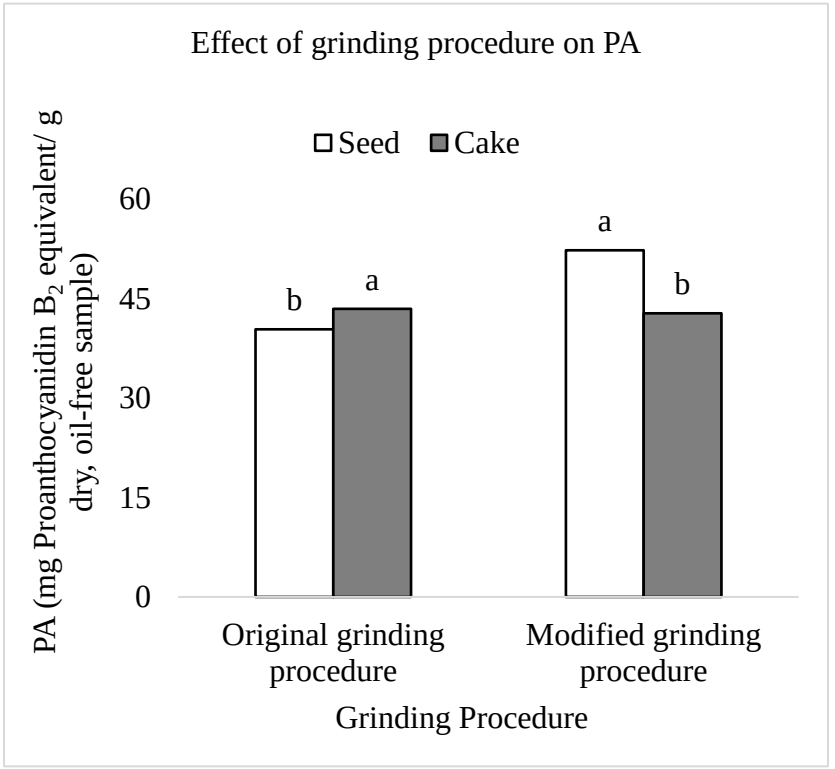

Fig 23. Comparison of grape seed and press cake in terms of PA with different grinding procedure. PA extracted from seeds and press cake of 'Cabernet Sauvignon' using 50% ethanol at pH 2 for 3 h of extraction (n = 3). Bar within grinding procedure with same group with different letters were significantly different ($p < 0.05$)

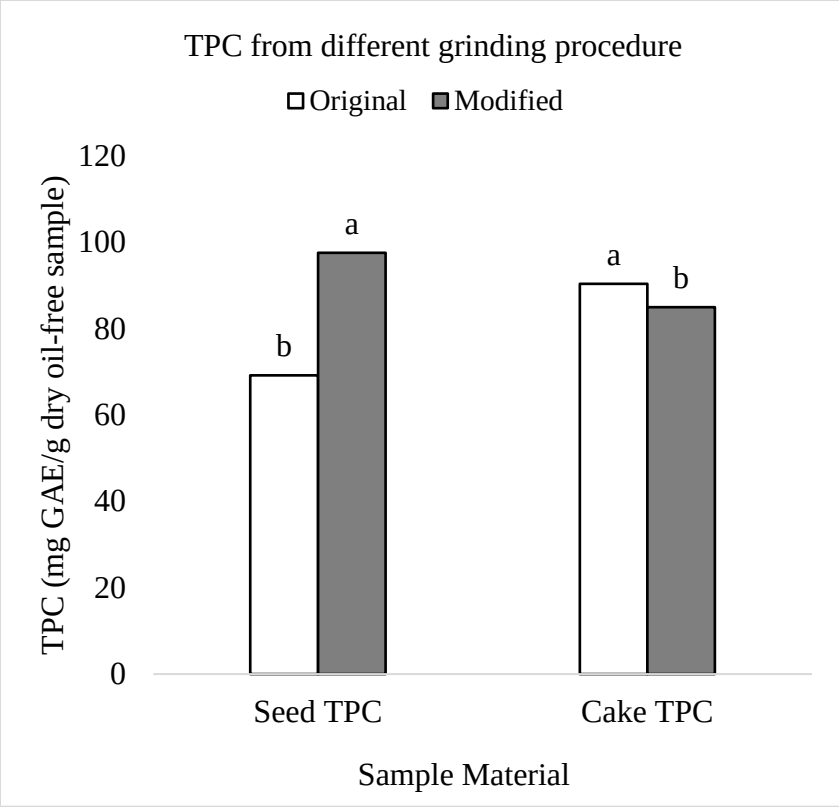

Fig 24. Effect of grinding procedure on TPC extraction yield from grape seed and press cake of 'Cabernet Sauvignon' extracted with 50% ethanol at pH 2 for 3 h of extraction (n = 3). Bar within grinding procedure with same group with different letters were significantly different ($p < 0.05$)

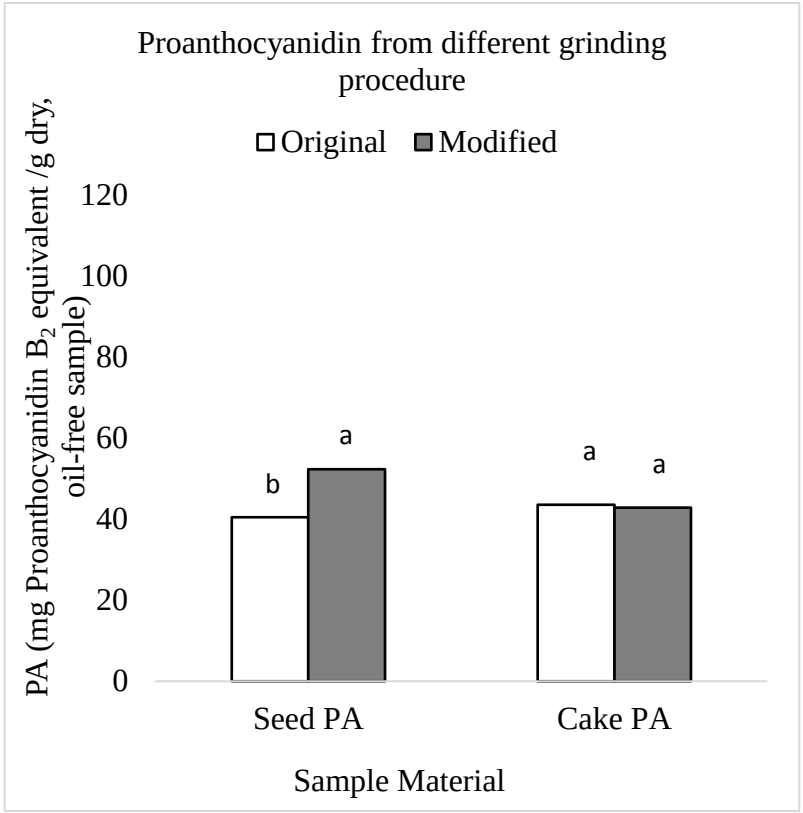

Fig 25. Effect of grinding procedure on PA extraction yield from grape seed and press cake of 'Cabernet Sauvignon' extracted with 50% ethanol at pH 2 for 3 h of extraction (n = 3). Bar within grinding procedure with same group with different letters were significantly different ($p < 0.05$)

4.3 Extraction of TPC and PA with Water

Grape seed flour has limited use in the food industry due to its objectionable astringency caused by proanthocyanidins (De Sa et al., 2014) present in the seeds. Partial extraction of polyphenols from grape seed flour could decrease the level of astringency and increase flour inclusion rate into food products (Hoye and Ross, 2011). Recovery of the extracted polyphenols could add value to the process with the extract having value as a dietary supplement. Extraction of phenolic compounds from grape seed flour using organic solvents is costly and incomplete recovery of the extraction solvent may yield a product which is unsafe for human health. Potential use of water for partial extraction of phenolic compounds from grape seed and press cake was studied given that water is inexpensive, environmentally friendly and safe for human body. Our study of TPC and PA extraction using water was conducted with three different pH levels (pH 5.4, pH 3 and pH 2) for 1 h, 3 h and 6 h of extraction at 80 ^{0}C. Grape seed and press cake of 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' were used in the study.

4.3.1 *Effect of time on TPC extraction from grape seeds and press cake using water as extraction solvent*

Extraction time was found to have a significant effect on TPC extraction from grape seeds and press cake with water as extraction solvent (Table 9). Total phenolics yield from seeds of each variety was significantly higher for extraction time above 1 h for each pH levels except from 'Sangiovese' seed and press cake. Extraction time had no significant difference in TPC yield from 'Sangiovese' press cake at pH 2 and from seeds at pH 3 and pH 5.4. Depending on the pH and grape variety, TPC at 6 h was either

significantly higher than 3 h or there was no significant difference in TPC yield between 3 h and 6 h. These results indicated that the longer extraction time (either 3 h or 6 h) favored TPC extraction. These results are similar to what was found for other organic solvents used in this study (Table 3). Lapornik et al. (2005) also extracted TPC from grape pomace using water and found significantly increased TPC yield from 1 h to 12 h.

Table 9. Effect of extraction time on TPC (mg GAE/g dry, oil-free sample) from 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' seed and press cake using water at different pH (pH 2, pH 3 and pH 5.4) (n = 3).

Variety	pH	Time	Seed		Press Cake	
			TPC	Sign [z]	TPC	Sign
Cabernet Sauvignon	5.4	1	10.62 c	*** [y]	14.20 b	**
		3	13.84 b		14.85 b	
		6	16.74 a		17.12 a	
	2	1	16.62 b	**	17.68 b	**
		3	17.84 b		18.13 b	
		6	21.05 a		20.33 a	
	3	1	11.54 c	***	13.79 b	**
		3	13.94 b		15.43 ab	
		6	17.29 a		16.91 a	
Merlot	5.4	1	20.00 b	***	19.22 b	**
		3	24.09 a		22.14 a	
		6	25.54 a		19.21 a	
	2	1	22.91 c	***	21.78 b	**
		3	29.94 b		25.02 a	
		6	32.46 a		23.53 a	
	3	1	18.89 b	***	19.09 b	**
		3	24.22 a		21.46 a	
		6	26.04 a		21.39 a	
Sangiovese	5.4	1	17.64 a [w]	NS [x]	18.57 a	*
		3	18.84 a		17.04 ab	
		6	20.27 a		16.26 b	
	2	1	16.84 b	*	19.49 a	NS
		3	19.35 a		18.50 a	
		6	19.61 a		18.15 a	
	3	1	14.44 a	NS	20.44 a	***
		3	16.05 a		16.52 b	
		6	17.04 a		16.77 b	

[z] Sign denotes significance.

[y] * Significance at α = 0.05, **. Significance at α = 0.01, *** Significance at α = 0.001.

[x] NS indicates no significant difference.

[w] Common letter within each column indicates no significant difference ($p < 0.05$).

4.3.2 *Effect of time on PA extraction from grape seeds and press cake using water as extraction solvent*

Effect of extraction time on PA yield from grape seed and press cake of different grape varieties at different pH levels of water is presented in Table 10. Unlike results found for TPC in which TPC yields increased with increase in extraction time, PA yields appeared to increase from 1 h to 3 h of extraction, but did not increase in most cases with an additional 3 h of time to 6 h of extraction. In a few cases, however, time was found to have no significant effect on PA extraction. Samples exhibiting no significant difference in PA yield with extraction time included 'Sangiovese' seed and press cake at pH 2, 'Sangiovese' press cake at pH 3 and 'Cabernet Sauvignon' press cake at pH 2. Extraction time of 3 h appeared to be sufficient for PA extraction using water as extraction solvent from press cake in many cases but 6 h was required for maximum extraction in other cases. This result is in accordance with our results with organic solvents where 3 h of extraction gave optimal PA extraction yield (Table 4).

We settled on 6 h of extraction time for further evaluations regarding effect of water pH on TPC and PA yield from grape seed and press cake.

Table 10. Effect of extraction time on PA (mg proanthocyanidin B_2 equivalent/g dry, oil-free sample) from 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' seed and press cake at different pH levels (5.4, 2 and 3) (n = 3).

Variety	pH	Time	Seed		Press Cake	
			PA	Sign [z]	PA	Sign
Cabernet Sauvignon	5.4	1	10.71 c	*** [y]	11.68 b	***
		3	13.23 b		13.38 a	
		6	15.76 a		13.92 a	
	2	1	14.85 b	*	13.56 a [w]	NS
		3	15.12 b		14.42 a	
		6	16.72 a		14.17 a	
	3	1	11.65 c	***	11.55 b	***
		3	13.34 b		13.33 a	
		6	16.29 a		14.09 a	
Merlot	5.4	1	18.31 c	***	16.08 c	***
		3	20.29 b		19.13 a	
		6	22.53 a		17.96 b	
	2	1	19.70 b	***	17.20 b	*
		3	21.93 a		18.72 a	
		6	22.95 a		18.30 a	
	3	1	17.33 c	***	15.99 b	***
		3	20.69 b		18.89 a	
		6	23.09 a		18.73 a	
Sangiovese	5.4	1	17.91 b	*	17.34 b	*
		3	19.81 a		19.03 a	
		6	19.98 a		18.10 ab	
	2	1	17.38 a	NS [x]	17.35 a	*
		3	18.85 a		17.40 a	
		6	18.48 a		15.89 b	
	3	1	16.54 b	**	17.90 a	NS
		3	18.58 a		18.56 a	
		6	18.87 a		17.91 a	

[z] Sign denotes significance.

[y] * Significance at $\alpha = 0.05$, **. Significance at $\alpha = 0.01$, *** Significance at $\alpha = 0.001$.

[x] NS indicates no significant difference.

[w] Common letter within each column indicates no significant difference ($p < 0.05$).

4.3.3 *Effect of pH on TPC extraction using water as extraction solvent*

Effect of water pH on TPC and PA extraction from grape seeds and press cake was evaluated at pH levels of 5.4 (water pH was unadjusted prior to extraction), 2 and 3 (Table 11). Total phenolic content extraction was significantly higher for water at pH 2 compared to that of pH 3 and pH 5.4 from seeds and press cake of all varieties except for 'Sangiovese'. There was no significant difference in TPC yield between pH 2 and pH 5.4 from 'Sangiovese' seed; acidity of water was found to have no significant effect on the TPC extraction from 'Sangiovese' press cake. The results showed that TPC extraction using water as solvent was favored by pH 2 in all cases when pH did influence TPC extraction yield. Effect of water pH on TPC extraction yield from grape seeds and press cake was similar to that of other organic solvents used in this study. With organic solvents used in this study, pH 2 favored TPC extraction when pH did influence TPC extraction (Table 1).

Table 11. Effect of water pH on the extraction of TPC (mg GAE/g dry, oil-free sample) and from 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' seeds and press cake for 6 h of extraction (n = 3).

Grape Variety	pH	Seed		Press cake	
		TPC	Sign [z]	TPC	Sign
Cabernet Sauvignon	5.4 2 3	16.7 b 21.0 a 17.3 b	*** [y]	17.1 b 20.3 a 16.9 b	***
Merlot	5.4 2 3	25.5 b 32.5 a 26.0 b	***	19.2 c 23.5 a 21.4 b	***
Sangiovese	5.4 2 3	20.3 a 19.6 a 17.0 b	*	16.3 a [w] 18.2 a 16.8 a	NS [x]

[z] Sign denotes significance.

[y] * Significance at α = 0.05, **. Significance at α = 0.01, *** Significance at α = 0.001.

[x] NS indicates no significant difference.

[w] Common letter within each column indicates no significant difference ($p < 0.05$).

4.3.4 *Effect of pH on PA extraction using water as extraction solvent*

Effect of water pH on PA extraction from grape seeds and press cake is presented in Table 12. Interestingly, no significant impact of water pH was observed in PA extraction yield from both grape seed and press cake of all grape varieties except for 'Sangiovese' press cake.

Table 12. Effect of acidity of water pH on the extraction of PA (mg/g dry, oil-free sample) from 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' seeds and press cake for 6 h of extraction (n = 3).

Grape Variety	pH	Seed		Press cake	
		PA	Sign [z]	PA	Sign
Cabernet Sauvignon	5.4 2 3	15.8 a [w] 16.7 a 16.3 a	NS [x]	13.9 a 14.2 a 14.1 a	NS
Merlot	5.4 2 3	22.5 a 23.0 a 23.1 a	NS	18.0 a 18.3 a 18.7 a	NS
Sangiovese	5.4 2 3	20.0 a 18.5 a 18.9 a	NS	18.1 a 15.9 b 17.9 a	*** [y]

[z] Sign denotes significance.

[y] * Significance at $\alpha = 0.05$, **. Significance at $\alpha = 0.01$, *** Significance at $\alpha = 0.001$.

[x] NS indicates no significant difference.

[w] Common letter within each column indicates no significant difference ($p < 0.05$).

The results indicated that water adjusted at pH 2 favors TPC extraction from seed and press cake, whereas proanthocyanidin extraction is probably insensitive to water acidity within the ranges tested. Similar results regarding PA extraction was found with 80% acetone in our study where in most of the cases, PA extraction from seeds and press cake with 80% acetone was not influenced by pH of 80% acetone (Table 2).

4.4 Varietal Comparison for TPC and PA Using Water as an Extraction Solvent

TPC and PA from grape seeds and press cake of three different Oklahoma grown varieties, 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' are shown in the Table 13. 'Merlot' seed had the highest TPC (32.5 mg GAE/g dry, oil-free seed), whereas TPC in 'Cabernet Sauvignon' (21 mg GAE/g dry oil-free seed) seed was equivalent to 'Sangiovese' (19.6 mg GAE/g dry, oil-free seed) seed. PA from grape seed of three different grape varieties were in the following order from the highest to the lowest: 'Merlot' (23. 5 mg proanthocyanidin B_2/g dry, oil-free seed) > 'Sangiovese' (18.2 mg proanthocyanidin B_2/g dry, oil-free seed) > 'Cabernet Sauvignon' (16.7 proanthocyanidin B_2/g dry, oil-free seed). Proanthocyanidin content in press cake was the highest for 'Merlot' (18.3 mg proanthocyanidin B_2/g dry, oil-free seed) followed by 'Sangiovese'

(15.9 mg proanthocyanidin B_2/g dry, oil-free seed) and 'Cabernet Sauvignon' (14.2 mg proanthocyanidin B_2/g dry, oil-free seed), respectively. Total phenolic extraction using 70% ethanol at pH 2 and PA extraction using 80% acetone at pH 2 in our study also showed the highest TPC and PA for 'Merlot' variety. Proanthocyanidin content of grape varieties using 80% acetone at pH 2 in our study was in the following order from the highest to the lowest: 'Merlot' > 'Sangiovese' > 'Cabernet Sauvignon', which is in accordance with the results found using water at pH 2 as extraction solvent.

Table 13. TPC (mg GAE/g dry, oil-free sample) and PA (mg proanthocyanidin B_2 equivalent/g dry, oil-free sample) in seeds and press cake of 'Cabernet Sauvignon', 'Merlot' and 'Sangiovese' extracted with water (pH 2) for 6 h of extraction time (n = 3).

Grape Variety	Seed				Press Cake			
	TPC	Sign [z]	PA	Sign	TPC	Sign	PA	Sign
Cabernet Sauvignon	21.0 b		16.7 c		20.3 b		14.2 c	
Merlot	32.5 a	*** [y]	23.0 a	***	23.5 a	***	18.3 a	***
Sangiovese	19.6 b		18.5 b		18.2 c		15.9 b	

[z] Sign denotes significance.

[y] *** Significance at $\alpha = 0.001$.

Water alone was found to be less efficient and recovered only about 20% of TPC compared to 70% ethanol at pH 2 and about 31% of PA from grape seed press cake compared to 80% acetone at pH 2 for 6 h of extraction (from Table 7 and 13). This finding is in accordance with the finding reported by Yilmaz and Toledo (2006) where

TPC extraction yield from ‘Muscadine’ seeds using water alone was about 20% compared to that of 70% ethanol. Our findings suggested that water alone could possibly be used for partial extraction of phenolic compounds, which might decrease the astringency of grape seed flour to the level which could be acceptable to a consumer. The inclusion rate which consumers find acceptable should be studied.

Although water alone could extract phenolic compounds which might decrease the astringency of grape seed flour to a level which could be acceptable to consumer, extraction time above 3 h might not be economically feasible for the commercial extraction. Extraction of TPC and PA using water was further studied, assisted with ultrasound technology, to evaluate the potential use of ultrasound to shorten the extraction time.

4.5 Ultrasound-assisted Extraction Using Water as Extraction Solvent

Ultrasound-assisted extraction with water was also investigated to explore the effect of ultrasound on increasing the solvent power of water to increase the TPC and PA yield observed for water alone. We settled on a 40:1 solvent to solids ratio because it agreed with previous recommendations for ultrasound-assisted extraction of 30:1 or 50:1 (Ghafoor et al., 2009; Zhang et al., 2011).

Prior to our study, preliminary tests were conducted to optimize UAE variables such as amplitude of the probe and water bath temperature needed to achieve a given extraction temperature. Four (40%, 60%, 80% and 100%) ultrasonic probe amplitude levels were tested with 50% ethanol (pH 2) at 80 ^{0}C for 20 min of sonication. Amplitude 100% was rejected because of excessive foaming in the sample/solvent mixture during

sonication, causing solid sample to aggregate above solvent level in the sample cup. Amplitude 80% was selected over 40% and 60% amplitude because TPC and PA yield appeared to be higher for 80% amplitude compared to 40% and 60% amplitude. Extraction temperature of 60 ^{0}C was selected over 80 ^{0}C due to the excessive evaporation of the solvent at 80 ^{0}C. Volume of the sample/solvent mixture sonicated at 80 ^{0}C was almost half of the sample/solvent volume sonicated at 60 ^{0}C. During sonication, cavitation bubbles form from the gas nuclei in the liquid which grow over time and burst violently generating very high local temperature of 5000 K (Soria and Villamiel, 2010). This heat release is constant and must be accounted for when regulating overall solvent temperature during sonication. A series of preliminary experiments were conducted to set the temperature of water bath at the specific temperature such that extraction solvent temperature was maintained at 60 ^{0}C during continuous sonication. The water bath supplied water to the jacketed glass cooling cell during sonication. The water bath was set at different temperatures and the extraction temperature during sonication was monitored using a thermocouple immersed in the sample/solvent mixture. Water bath temperatures which maintained the sample/solvent mixture temperature at 60 ^{0}C during sonication for a given time were selected and are shown in Table 14.

Table 14. Water bath temperature setting to maintain solvent extraction temperature at 60 ^{0}C for different extraction durations.

Time (minutes)	Water bath temperature (^{0}C)	Extraction solvent
5, 10, 20, 40, 60, 180, 360	48	Water (pH 2) and preheated water (pH 2) at 60 ^{0}C
0.5, 1, 2	56	Pre heated water (pH 2) at 60 ^{0}C

Grape seed press cake of 'Sangiovese' variety ground using our modified grinding procedure was used as sample material. Extractions were conducted with water at pH 2 for durations of 30 s, 1 min, 2 min, 5 min, 10 min, 20 min, 40 min, 1 h, 2 h and 3 h. Table 15 shows the ANOVA for the ultrasound-assisted extraction (UAE) conducted using water (pH 2) at 60 ^{0}C for 5 min, 10 min, 20 min, 40 min, 1 h, 2 h and 3 h duration.

Table 15. ANOVA table for ultrasound-assisted extraction and conventional extraction of TPC and PA with water (pH 2) from 'Sangiovese' grape seed press cake extracted at 60 ^{0} C.

Effect	Df	TPC (Sign) [z]	PA (Sign)
Treatment	1	NS [x]	NS
Time	6	*** [y]	***
Treatment × Time	6	***	*

[z] Sign denotes significance.

[y] * Significance at α = 0.05, ** Significance at α = 0.01*** Significance at α = 0.001.

[x] NS indicates not significant.

Treatment (UAE and conventional extraction) had no significant impact on TPC and PA extraction. Time and interaction of time with treatment had significant impact on TPC and PA yield (Table 15)

Total phenolic content was significantly higher for UAE compared to conventional extraction up to 10 min and no significant difference in TPC yield was found between UAE and conventional extraction from 20 min to 60 min (Fig 26). For extraction time above 1 h, TPC yield was significantly higher for conventional extraction. There was no significant difference between 3 h of conventional extraction and 40 min of UAE in terms of TPC yield. Pingret et al. (2012) found 40 min of UAE at 40 ^{0} C using

water to be optimum for phenolic compound extraction from apple pomace. Other studies have found that TPC extraction from plant tissues including grape seeds with UAE significantly reduces the extraction time compared to that of conventional extraction (Da Porto et al., 2013; Khan et al., 2010).

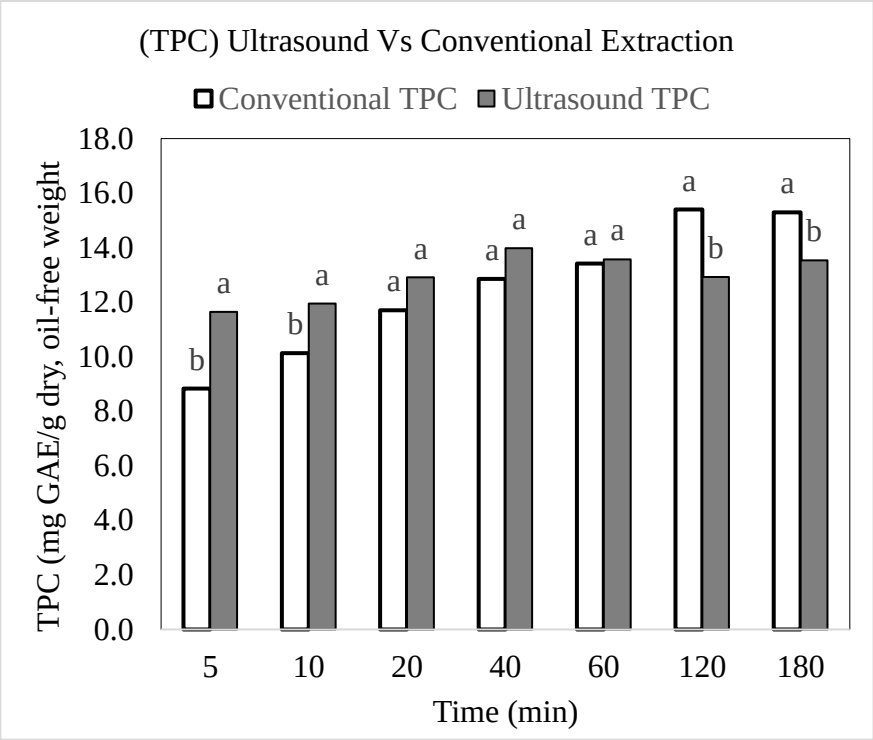

Fig. 26. Ultrasound-assisted extraction versus conventional extraction of TPC (mg GAE/g dry, oil-free sample) from press cake of 'Sangiovese' grape variety extracted using water (pH 2) extracted at 60 ^{0} C (n = 3). Common letter within each pair of bars indicates no significant difference ($P < 0.05$)

Proanthocyanidin was significantly higher for UAE compared to that of conventional runs at 5 min and no significant difference between UAE and conventional extraction was found between UAE and conventional extraction with increased time up to 2 h. For 3 h of extraction, conventional extraction gave significantly higher PA compared to UAE (Fig 27). Proanthocyanidins yield appeared to increase with sonication time from

5 min to 40 min and remained almost steady after 40 min indicating that longer sonication time might not be feasible in increasing PA extraction yield. PA yield for 1 h of UAE was found equivalent to 3 h of conventional extraction.

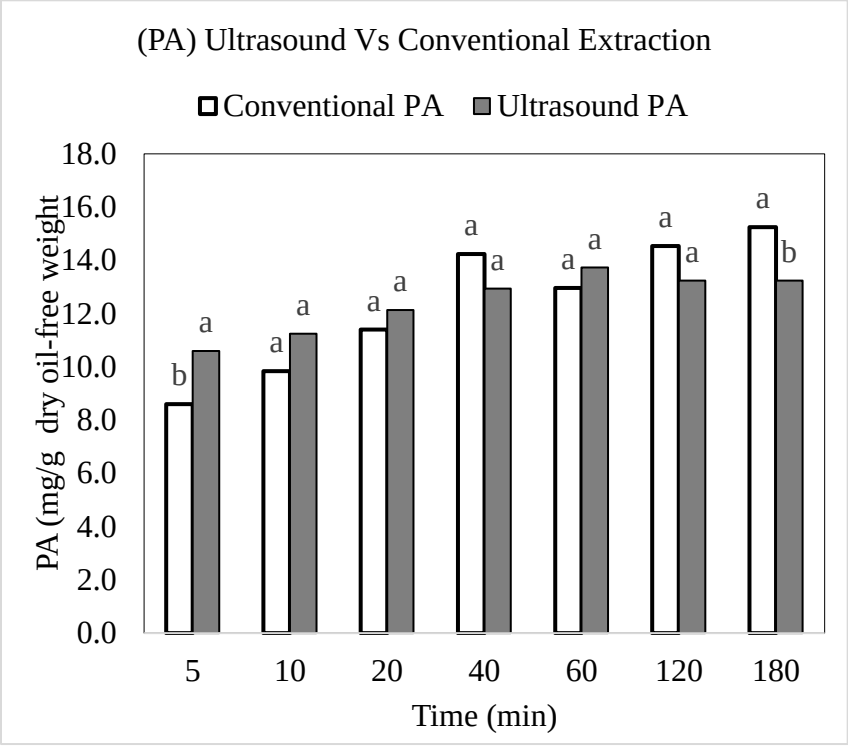

Fig. 27. Ultrasound-assisted extraction versus conventional extraction of PA (mg Proanthocyanidin B_2 equivalent/g dry, oil-free sample) from press cake of 'Sangiovese' grape variety extracted using water (pH 2) extracted at 60 ^{0}C (n = 3). Common letter within each pair of bars indicates no significant difference ($P < 0.05$).

Our findings indicated that UAE significantly decreases TPC and PA extraction time compared to that of conventional extraction. Higher TPC and PA for conventional extraction compared to that of UAE at times above 1 h could be due to degradation of phenolic compounds. To get a better insight on the effect of ultrasound on TPC and PA extraction from grape seed press cake, UAE was repeated at 60 ^{0}C for 30 s, 1 min , 2 min,

5 min , 10 min, 20 min, 40 min and 1 h duration. Treatment (UAE and conventional extraction) and extraction time was found significant for TPC and PA. Interaction of treatment and time was found to be significant for TPC but not significant for PA (Table 16).

Table 16. ANOVA table for ultrasound-assisted extraction and conventional extraction of TPC and PA with pre-heated water (pH 2) from 'Sangiovese' grape seed press cake extracted at 60 ^{0} C.

Effect	Df	TPC (Sign) [z]	PA (Sign)
Treatment	1	*** [y]	***
Time	7	***	***
Treatment × Time	7	**	NS [x]

[z] Sign denotes significance.

[y] * Significance at α = 0.05, ** Significance at α = 0.01*** Significance at α = 0.001.

[x] NS indicates not significant.

The results showed that TPC was significantly higher for ultrasound-assisted extraction compared to conventional extraction at all extraction durations except for 5 min of extraction (Fig. 28). It was found that UAE for 30 s yielded TPC equivalent to 1 h of conventional extraction (Table 17). After 5 min up to 20 min, TPC yield using UAE

was equivalent to 1 h of conventional extraction. After 20 min, TPC yield using UAE was significantly higher than 1 h of conventional extraction (Table 17).

Table 17. Comparison of TPC (mg GAE/g dry, oil-free sample) and PA (mg proanthocyanidin B_2 equivalent/ g dry, oil-free sample) between 1h of conventional extraction at each level of UAE.

Comparison	TPC (Sign [z])	PA (Sign)
Conventional 1h Vs UAE 30 seconds	NS	* [y] (C > U)
Conventional 1h Vs UAE 1 minute	* (C > U)	NS [x]
Conventional 1h Vs UAE 2 minute	*** (C > U)	NS
Conventional 1h Vs UAE 5 minute	* (C > U)	NS
Conventional 1h Vs UAE 10 minute	NS	NS
Conventional 1h Vs UAE 20 minute	NS	NS
Conventional 1h Vs UAE 40 minute	** (U > C)	** (U > C) [w]
Conventional 1h Vs UAE 1 h	** (U > C)	** (U > C)

[z] Sign denotes significance.

[y] * Significance at $\alpha = 0.05$, ** Significance at $\alpha = 0.01$.

[x] NS indicates not significant.

[w] Letter 'U' indicates ultrasound-assisted extraction and the letter 'C' indicates conventional extraction.

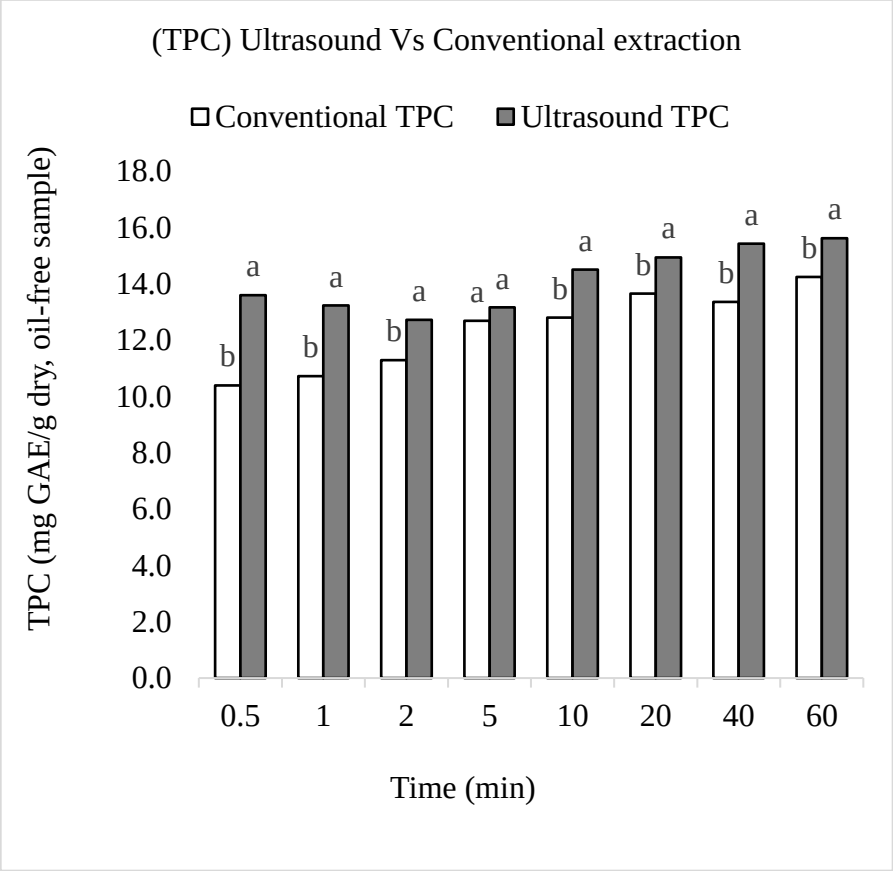

Fig 28. Ultrasound-assisted extraction versus conventional extraction of TPC (mg GAE/g dry, oil-free sample) from press cake of 'Sangiovese' grape variety extracted using pre-heated water (pH 2) at 60 ^{0} C (n = 3). Common letter within each pair of bars indicates no significant difference ($P < 0.05$).

The results showed that the PA was significantly higher for ultrasound-assisted extraction compared to conventional extraction at each levels of extraction time (Fig. 29).

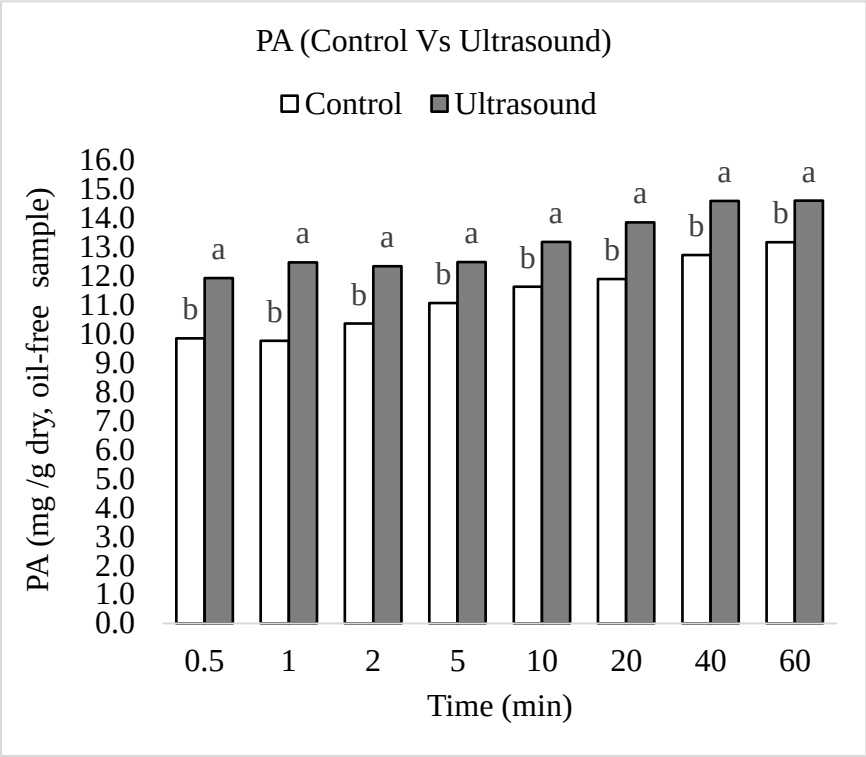

Fig 29. Ultrasound-assisted extraction versus conventional extraction of PA (mg proanthocyanidin B_2 equivalent/g dry, oil-free sample) from press cake of 'Sangiovese' grape variety extracted using pre-heated water (pH 2) at 60 ^{0}C (n = 3). Common letter within each pair of bars indicates no significant difference ($P < 0.05$).

There was no significant difference in PA yield between 1 min of UAE and 1h of conventional extraction (Table 17). Similar trend was found up to 20 min of extraction. After 20 min, PA yield using UAE was significantly higher than 1 h of conventional extraction. Although further studies might be required to validate that the TPC and PA extraction yield for 1 min using UAE would always be equivalent to 1 h of conventional extraction, it can be concluded that ultrasound significantly reduces the extraction time compared to that of 1 h of conventional extraction. With UAE, TPC and PA yield appeared to reach a plateau after 10 to 20 min suggesting that the longer extraction time above 20 min do not increase the yield. However, TPC and PA yield with conventional extraction appeared to increase with the extraction time (Fig. 30 and 31).

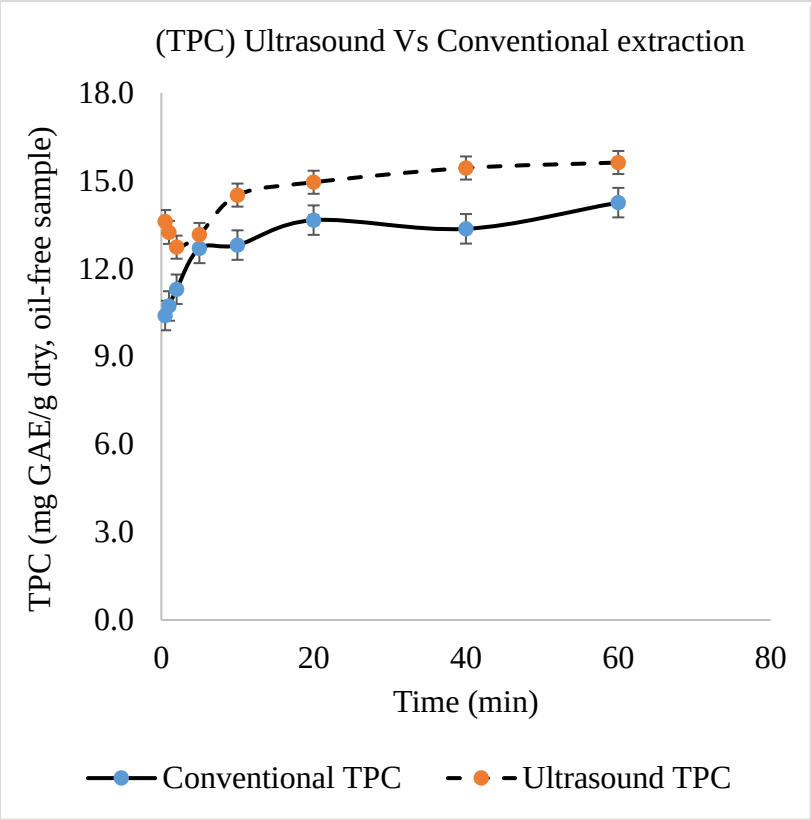

Fig 30. Ultrasound-assisted extraction versus conventional extraction of TPC (mg GAE/g dry, oil-free sample) from press cake of 'Sangiovese' grape variety extracted using pre-heated water (pH 2) at 60 ^{0} C. Error bars show maximum and minimum value of TPC (n = 3). ($P < 0.05$).

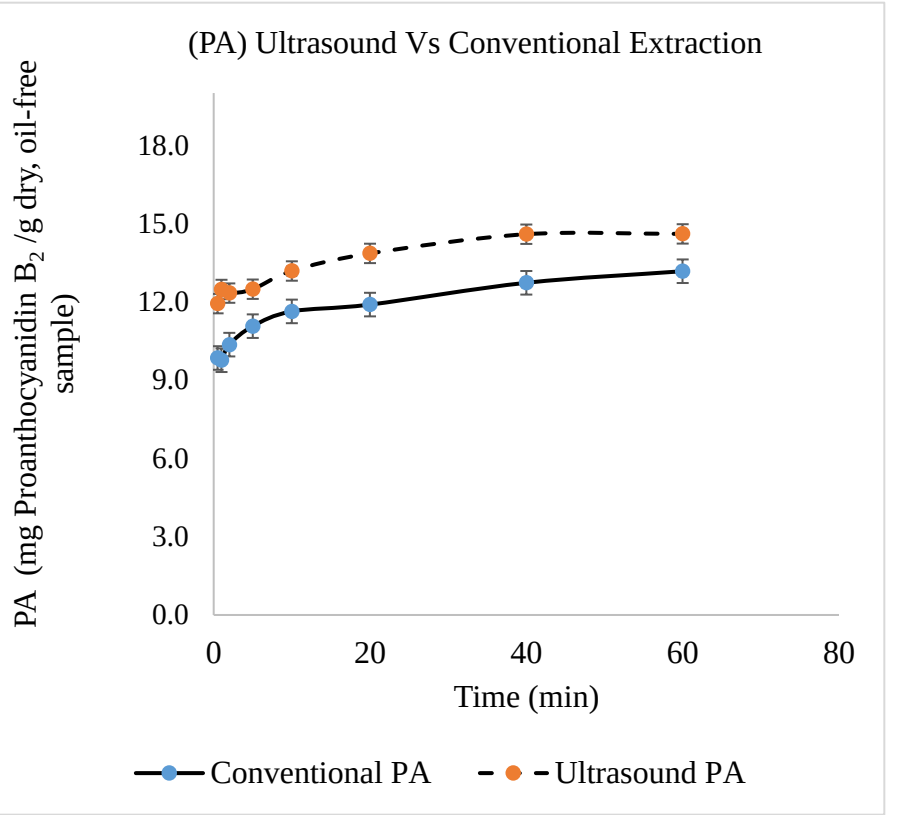

Fig 31. Ultrasound-assisted extraction versus conventional extraction of PA (mg proanthocyanidin B_2/g dry, oil-free sample) from press cake of 'Sangiovese' grape variety extracted using pre-heated water (pH 2) at 60 ^{0} C. Error bars show maximum and minimum value of TPC (n = 3). ($P < 0.05$).

Results from this study suggested that the shorter extraction time of 10 minute was equivalent in terms of total phenolic content and PA from grape seed press cake compared to conventional extraction for 1 h using water as a partial extraction solvent. TPC and PA yield at 1 h from 'Sangiovese' press cake using UAE with water (pH 2) as extraction solvent was significantly lower than that of the analytical extraction conducted for 'Sangiovese' press cake using 80% acetone (pH 2) for 3 h. Total phenolic content and PA yield from 'Sangiovese' press cake using 80% acetone at pH 2 for 3 h were 66.7 mg GAE/g dry, oil-free sample and 45.5 mg/g dry, oil-free sample, respectively.

Ultrasound-assisted extraction has been reported to significantly reduce the polyphenol extraction time from different plant materials under varying extraction conditions (Carrera et al., 2012; Khan et al., 2010). Extraction of total phenolic yield using water at $25 \pm 2\ ^{0}$C from pomegranate peel was reduced to 6 min with UAE compared to 1 h of conventional extraction (Pan et al., 2012). Our results suggested that the maximum recovery of phenolic compounds using UAE may have been completed within 10 min. It was found that UAE for 1 h increased TPC (Fig. 28) and PA (Fig. 29) yield by 9% and 10% respectively compared to conventional extraction for 1 h. This finding is in agreement with Vilkhu et al. (2008) who reported polyphenolic yield increase up to 6% to 35% from red grape marc using UAE.

Findings of UAE in this study suggested that UAE is better than conventional extraction by increasing the TPC and PA content and/or by reducing the extraction time. The combination of UAE for a short time of 10 min followed by conventional extraction could maximize the extraction efficiency.

CHAPTER V

CONCLUSION

All factors (solvent type, solvent pH and extraction time) considered in this study were found to influence TPC and PA extraction from grape seeds and press cake. The influence of pH 2 solvent adjustment over pH 3 probably favors TPC and PA extraction yield. Interestingly, proanthocyanidin extraction yield appeared to be less influenced by pH of 80% acetone. With organic solvents, our study demonstrated that extraction time of 6 h was probably required for the maximum recovery of TPC whereas PA could be optimally extracted within 3 h of extraction. TPC extraction studies for longer time above 6 h and PA extraction studies with extraction time between 1h and 3h could be useful in optimizing TPC and PA extraction duration. Insensitivity of PA extraction to acidity of 80% acetone could be studied further to investigate the reasons why PA extraction appeared to be less sensitive to solvent acidity. Ethanol (70%) adjusted at pH 2 was a better extraction solvent for TPC, while PA extraction yield was favored by 80% acetone. Further studies related to the impact of solvent polarity on phenolic compound extraction kinetics might provide better insight in this regard. Optimal conditions for TPC and PA

extraction obtained in this study could be studied further to evaluate the extractability of individual phenolic compounds using HPLC.

Seeds of Oklahoma grown grape varieties: ‘Cabernet Sauvignon’, ‘Merlot’ and ‘Sangiovese’ were found to be rich in phenolic compounds accounting for 8.3% to 9.4% TPC and 4.3% to 6.8% PA of the seed weight on a dry, oil-free weight basis. Our TPC concentration was higher than the 5% to 8% documented previously, but while our data corrected for varying oil contents of different grape varieties prior to and after oil pressing, other studies did not. Total phenolic content from grape seeds in our study without correction for oil content ranged from 7.3% to 8.4%. Among three different varieties studied, ‘Merlot’ had the highest TPC and PA content.

Total phenolic content and PA extraction yield using acidified water as extraction solvent were found highest at 6 h and 3 h of extraction, respectively. However, extraction studies for TPC above a 6h extraction duration and for PA with extraction durations ranging from 3h to 6h could give better precision related to the extraction time required for maximum yields. Insensitivity of water acidity to PA extraction could also be studied further. Water at pH 2 was able to recover a modest amount of phenolics from grape seed, about 20% TPC and 31% PA, compared to that of organic solvents. Although our study demonstrated that water could potentially be used for partial extraction of phenolic compounds which could reduce the astringency of grape seed flour, from the data collected it was not possible to determine if the decreased astringency of grape seed flour could be acceptable to consumers. Further studies are therefore necessary to determine the inclusion rate of grape seed flour in food products which consumers find acceptable.

Ultrasound-assisted extraction of TPC and PA from grape seed press cake with water showed that ultrasound-assisted extraction not only reduced the extraction time to 10 min but also increased TPC and PA recovery by 10% compared to 1 h of conventional extraction with acidified water solvent. Our results indicated that UAE at our extraction temperature of 60 ^{0}C substantially increased yield into water as solvent within the first 10 minutes of the extraction cycle. Further studies might be required to validate whether TPC and PA extraction for times shorter than 5 min with UAE might be equivalent to 1 h of conventional extraction. Combination of UAE for shorter time followed by conventional extraction could be investigated further with water and organic solvents to explore whether the combination of UAE and conventional extraction could improve the phenolic compounds extraction yield within shorter extraction time compared to conventional extraction alone. Flow-through ultrasound-assisted extraction could be studied to evaluate its potential use in large scale commercial extraction of phenolic compounds.

REFERENCES

Arts, I.C. and P.C. Hollman. 2005. Polyphenols and disease risk in epidemiologic studies. Amer. Soc. Clinical. Nutr. 81:317-325.

Arvanitoyannis, I.S., D. Ladas, and A. Mavromatis. 2006. Potential uses and applications of treated wine waste: a review. Intl. J. Food Sci. Technol. 41:475-487.

Bavaresco, L. 2003. Role of viticultural factors on stilbene concentrations of grapes and wine. Drugs. Expt. Clinical. Res. 29:181-187.

Bennett, R.N. and R.M. Wallsgrove.1994. Secondary metabolites in plant defence mechanisms. New. Phytol. 127:617-633.

Bozan, B., G. Tosun, and D. Ozcan. 2008. Study of polyphenol content in the seeds of red grape (*Vitis vinifera* L.) varieties cultivated in Turkey and their antiradical activity. Food Chem. 109:426-430.

Brigitte A.G., P.E. Milbury, and J.B. Blumberg. 2005. Flavonols, Flavones, Flavanones, and Human Health: Epidemiological Evidence. J. Medicianal Food. 8:281-290.

Bucić-Kojić, A., M. Planinić, S. Tomas, M. Bilić, and D. Velić, 2007. Study of solid–liquid extraction kinetics of total polyphenols from grape seeds. J. Food Eng. 81:236-242.

Carrera, C., A. Ruiz-Rodriguez, M. Palma, and C.G. Barroso. 2012. Ultrasound assisted extraction of phenolic compounds from grapes. Anal. Chem. Acta. 732:100-104.

Chapman, D.J. and M. Ragan. 1980. Evolution of biochemical pathways: evidence from comparative biochemistry. Annu. Rev. Plant. Phys. 31:639-678.

Cheng, V.J., A.E.-D.A. Bekhit, M. McConnell, S. Mros, and J. Zhao. 2012. Effect of extraction solvent, waste fraction and grape variety on the antimicrobial and antioxidant activities of extracts from wine residue from cool climate. Food Chem. 134:474-482.

Cheynier, V. 2005. Polyphenols in foods are more complex than often thought. Amer. J. Clinical. Nutr. 81:223S-229S.

Corinne P, J. Rigaud, V. Cheynier, and M. Moutounet. 1994. Oligomeric and polymeric procyanidins from grape seeds. Phytochemistry 36:781-784.

Corrales, M., A.F. García, P. Butz, and B. Tauscher. 2009. Extraction of anthocyanins from grape skins assisted by high hydrostatic pressure. J. Food Eng. 90:415-421.

Croteau, R., T.M. Kutchan, and N.G. Lewis. 2000. Natural products (secondary metabolites). Biochem. Mol Biol. Plants. 24:1250-1319.

Da Porto, C., E. Porretto, and D. Decorti. 2013. Comparison of ultrasound-assisted extraction with conventional extraction methods of oil and polyphenols from grape (*Vitis vinifera* L.) seeds. Ultrasonics Sonochem. 20:1076-1080.

Da Silva, J.M.R., J. Rigaud, V. Cheynier, A. Cheminat, and M. Moutounet. 1991. Proanthocyanidin dimer and trimers from grape seeds. Phytochemistry 30:1259-1264.

De Campos, L.M., F.V. Leimann, R.C. Pedrosa, and S.R. Ferreira. 2008. Free radical scavenging of grape pomace extracts from Cabernet Sauvingnon (*Vitis vinifera*). Bioresource Technol. 99:8413-8420.

De Sa, M., V. Justino, M.I. Spranger, Y.Q. Zhao, L. Han, and B.S. Sun. 2014. Extraction yields and anti-oxidant activity of proanthocyanidins from different parts of grape pomace: effect of mechanical treatments. Phytochemical Anal. 25:134-140.

Fine, A.M. 2000. Oligomeric Proanthocyanidin Complexes: History, Structure, and Phytopharmaceutical Applications. Alternative Medicine Rev. 5:144-.

Fontana, A.R., A. Antoniolli, and R. Bottini. 2013. Grape pomace as a sustainable source of bioactive compounds: extraction, characterization, and biotechnological applications of phenolics. J. Agr Food Chem. 61:8987-9003.

Freitas, V.A.P.D., Y. Glories, G. Bourgeoi, and C. Vitry. 1998. Characterisation of oligomeric and polymericprocyanidins from grape seeds by liquid secondary ion mass spectrometry. Phytochemistry 49:1435-1441.

Gao, L. and G. Mazza. 1995. Characterization, quantitation, and distribution of anthocyanins and colorless phenolics in sweet cherries. J. Agr. Food Chem. 43:343-346.

Ghafoor, K., Y.H. Choi, J.Y. Jeon, and I.H. Jo. 2009. Optimization of ultrasound-assisted extraction of phenolic compounds, antioxidants, and anthocyanins from grape (*Vitis vinifera*) seeds. J. Agr. Food Chem. 57:4988-4994.

Godjevac, D., V. Tesevic, M. Velickovic, L. Vujisic, V. Vajs, and S. Milosavljevic. 2010. Polyphenolic compounds in seeds from some grape cultivars grown in Serbia. J. Serbian. Chem Soc. 75:1641-1652.

Göhl, B. 1982. Les Aliments du bétail sous les tropiques: données sommaires et valeurs nutritives. Organisation des Nations Unies pour l'alimentation et l'agriculture.

Haddock, E.A., R.K. Gupta, S.M.K. Al-Shafi, K. Layden, E. Haslam, and D. Magnolato. 1982. The metabolism of gallic acid and hexahydroxydiphenic acid in plants: biogenetic and molecular taxonomic considerations. Phytochemistry 21:1049-1062.

Hoye, C. and C. Ross. 2011. Total phenolic content, consumer acceptance, and instrumental analysis of bread made with grape seed flour. J. Food Sci. 76:S428-S436.

Huh, Y.S., T.H. Hong, and W.H. Hong. 2004. Effective extraction of oligomeric proanthocyanidin (OPC) from Wild Grape Seeds. Biotechnol. Bioprocess Eng. 9:471-475.

Ignat, I., I. Volf, and V.I. Popa. 2011. A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. Food Chem. 126:1821-1835.

Yamakoshi, J., M. Saito, S. Kataoka, and M. Kikuchi. 2002. Safety evaluation of proanthocyanidin-rich extract from grape seeds. Food Chem. Toxicology 40:599-607.

Jayaprakasha, G., R. Singh, and K. Sakariah. 2001. Antioxidant activity of grape seed (Vitis vinifera) extracts on peroxidation models in vitro. Food Chem. 73:285-290.

Ju, Z.Y. and L.R. Howard. 2003. Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin. J. Agr Food Chem. 51:5207-5213.

Karvela, E., D.P. Makris, N. Kalogeropoulos, and V.T. Karathanos. 2009a. Deployment of response surface methodology to optimise recovery of grape (*Vitis vinifera*) stem polyphenols. Talanta 79:1311-1321.

Karvela, E., D.P. Makris, N. Kalogeropoulos, V.T. Karathanos, and P. Kefalas. 2009b. Factorial design optimisation of grape (*Vitis vinifera*) seed polyphenol extraction. European Food Res. Technol. 229:731-742.

Khan, M.K., M. Abert-Vian, A.-S. Fabiano-Tixier, O. Dangles, and F. Chemat. 2010. Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (*Citrus sinensis* L.) peel. Food Chemistry 119:851-858.

Konczaka, I. and W. Zhang. 2004. Anthocyanins—More ThanNature's Colours. J. Biomedicine. Biotechnol. 239-240.

Lapornik, B., M. Prošek, and A. Golc Wondra. 2005. Comparison of extracts prepared from plant by-products using different solvents and extraction time. J. Food Eng. 71:214-222.

Lattanzio, V. 2013. Phenolic compounds: introduction, p. 1543-1580, Natural Products. Springer.

Li, Y., G.K. Skouroumounis, G.M. Elsey, and D.K. Taylor. 2011. Microwave-assistance provides very rapid and efficient extraction of grape seed polyphenols. Food Chem. 129:570-576.

Lin, F. and M.M. Giusti. 2005. Effect of solven polarity and acidity on the extraction efficiency of isoflavones from soyabeans (*Glycine max*). J. Agri. Food Chem. 53:3795- 800.

Liu, S.X. and E. White. 2012. Extraction and characterization of proanthocyanidins from grape seeds.The Open Food Sci. J. 6:5-11.

M. Librán, C., L. Mayor, E. M. Garcia-Castello, and D. Vidal-Brotons. 2013. Polyphenol extraction from grape wastes: Solvent and pH effect. Agr. Sci. 04:56-62.

Macheix, J.-J. and A. Fleuriet. 1990. Fruit phenolics. CRC press.

Maier, T., A. Schieber, D.R. Kammerer, and R. Carle. 2009. Residues of grape (Vitis *vinifera* L.) seed oil production as a valuable source of phenolic antioxidants. Food Chem.112:551-559.

Makris, D.P., G. Boskou, and N.K. Andrikopoulos. 2007. Recovery of antioxidant phenolics from white vinification solid by-products employing water/ethanol mixtures. Bioresource Technol. 98:2963-2967.

Maqsood, S., S. Benjakul, and F. Shahidi. 2013. Emerging role of phenolic compounds as natural food additives in fish and fish products. Critical. Rev. Food Sci. Nutr. 53:162-179.

Mason, T.J., L. Paniwnyk, and J.P. Lorimer. 1996. The use of ultrasound in food technology. Ultrasonics Sonochem. 3:S254-S260.

Mastromatteo, M., A. Conte, and M.A. Del Nobile. 2010. Combined use of modified atmosphere packaging and natural compounds for food preservation. Food Eng. Rev. 2:28-38.

McClements, D.J. 1995. Advances in the application of ultrasound in food analysis and processing. Trends in Food Sci. Technol. 6:293-299.

Mennen, L.I., D. Sapinho, A.D. Bree, N. Arnault, S. Bertrais, P. Galan, and A.S. Hercberg. 2004. Consumption of foods rich in flavonoids is related to a decreased cardiovascular risk in apparently healthy french women. Amer. Soc. Nutr Sci. 134.

Merken, H.M. and G.R. Beecher. 2000. Measurement of food flavonoids by high-performance liquid chromatography: a review. J. Agri. Food Chem. 48:577-599.

Musee, N., L. Lorenzen, and C. Aldrich. 2007. Cellar waste minimization in the wine industry: a systems approach. J. Cleaner Prod. 15:417-431.

Mylonaki, S., E. Kiassos, D.P. Makris, and P. Kefalas. 2008. Optimisation of the extraction of olive (*Olea europaea*) leaf phenolics using water/ethanol-based solvent systems and response surface methodology. Anal. Bioanal. Chem. 392:977-985.

Nagels, L., V.D. W, J.D. Brucker, and H.D. Pooter. 1980. High-performance liquid chromatographic separation of naturally occurrin G esters of phenolic acids. J. Chromatography. 187:181-187.

Northup, R.R., R.A. Dahlgren, and J.G. McColl. 1998. Polyphenols as regulators of plant-litter-soil interactions in northern California's pygmy forest: a positive feedback? Biogeochem. 42:189-220.

Palma, M., Z. Pineiro, and C.G. Barroso. 2001. Stability of phenolic compounds during extraction with superheated solvents. J. Chromatography *A* 921:169-174.

Pan, G., G. Yu, C. Zhu, and J. Qiao. 2012. Optimization of ultrasound-assisted extraction (UAE) of flavonoids compounds (FC) from hawthorn seed (HS). Ultrasonics sonochem. 19:486-490.

Pandey, K.B. and S.I. Rizvi. 2009. Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Medicine and Cellular Longevity. 2:270-278.

Panouillé, M., M. Ralet, E. Bonnin, and J. Thibault. 2007. Recovery and reuse of trimmings and pulps from fruit and vegetable processing. Woodhead Publishing Limited: Cambridge, UK.

Pantelic, M.M., D.C. Dabic Zagorac, S.M. Davidovic, S.R. Todic, Z.S. Beslic, U.M. Gasic, Z. Tesic, and M.M. Natic. 2016. Identification and quantification of phenolic compounds in berry skin, pulp, and seeds in 13 grapevine varieties grown in Serbia. Food Chem. 211:243-252.

Parmas, G., M. Ana, Ana. M, S. Esteban-Ruano, C. Santos-Buelga, S. De Pascual-Teresa, J.N.C, and R. Gonzalo. 2004. Flavanol content and antioxidant activity in winery byproducts. J. Agr. Food Chem. 52:234-238.

Pekić, B., V. Kovač, E. Alonso, and E. Revilla. 1998. Study of the extraction of proanthocyanidins from grape seeds. Food Chem. 61:201-206.

Pingret, D., A.S. Fabiano-Tixier, C.L. Bourvellec, C.M.G.C. Renard, and F. Chemat. 2012. Lab and pilot-scale ultrasound-assisted water extraction of polyphenols from apple pomace. J. Food Eng. 111:73-81.

Porter, L., P. Dey, and J. Harborne. 1989. Methods in plant biochem. Methods in plant biochemistry: Plant phenolics. vol 1. Academic Press, San Diego, London.

Prior, R.L., E. Fan, H. Ji, A. Howell, C. Nio, M.J. Payne, and J. Reed. 2010. Multi-laboratory validation of a standard method for quantifying proanthocyanidins in cranberry powders. J. Sci. Food Agr. 90:1473-1478.

Quideau, S., D. Deffieux, C. Douat-Casassus, and L. Pouysegu. 2011. Plant polyphenols: chemical properties, biological activities, and synthesis. Angewandte Chemie 50:586-621.

Revilla, E., E. Alonso, and V. Kovac. 1995. The content of catechins and procyanidins in grapes and wines as affected by agroecological factors and technological practices. Amer Chem Soc. 69-80.

Robbins, R.J. 2003. Phenolic acids in foods: An overview of analytical methodology. J. Agr. Food Chem. 51:2866-2887.

Ross, K.A., T. Beta, and S.D. Arntfield. 2009. A comparative study on the phenolic acids identified and quantified in dry beans using HPLC as affected by different extraction and hydrolysis methods. Food Chem. 113:336-344.

Ruggieri, L., E. Cadena, J. Martínez-Blanco, C.M. Gasol, J. Rieradevall, X. Gabarrell, T. Gea, X. Sort, and A. Sánchez. 2009. Recovery of organic wastes in the Spanish wine industry. Technical, economic and environmental analyses of the composting process. J. Cleaner Prod. 17:830-838.

Saleem, M., H.J. Kim, M.S. Ali, and Y.S. Lee. 2005. An update on bioactive plant lignans. Natural Product Reports. 22:696-716.

Sant'Anna, V., A. Brandelli, L.D.F. Marczak, and I.C. Tessaro. 2012. Kinetic modeling of total polyphenol extraction from grape marc and characterization of the extracts. Separation and Purification Technol. 100:82-87.

Savova, M., T. Kolusheva, A. Stourza, and I. Seikova. 2007. The use of group contribution method for predicting the solubility of seed polyphenols of vitis vinifera L. within a wide polarity range in solvent mixtures. J. Univ. Chem Technol. Metallurgy 42:295-300.

Scalbert, A., C. Manach, C. Morand, C. Remesy, and L. Jimenez. 2005. Dietary polyphenols and the prevention of diseases. Critical Rev. Food Sci. Nutr. 45:287-306.

Schieber, A., D. Müller, G. Röhrig, and R. Carle. 2002. Effects of grape cultivar and processing on the quality of cold-pressed grape seed oils. Mitteilungen Klosterneuburg. 52:29-33.

Shi, J., J. Yu, J. Pohorly, J.C. Young, M. Bryan, and Y. Wu. 2003a. Optimization of the extraction of polyphenols from grape seed meal by aqueous ethanol solution. Food. Agri. Environ. 1:42-47.

Shi, J., J. Yu, J.E. Pohorly, and Y. Kakuda. 2003b. Polyphenols in grape seed-Biochemistry and Functionality. J. Medicianal Food. 6:291-299.

Shi, P., T. Yue, L. Ai, Y. Cheng, J. Meng, M. Li, and Z. Zhang. 2016. Phenolic compound profiles in grape skins of Cabernet Sauvignon, Merlot, Syrah and

Marselan cultivated in the Shacheng area (China). South African J. Enol. Viticult. 37:132-138.

Soria, A.C. and M. Villamiel. 2010. Effect of ultrasound on the technological properties and bioactivity of food: a review. Trends Food Sci. Technol. 21:323-331.

Spencer, J.P., M.M. Mohsen, A.M. Minihane, and J.C. Mathers. 2008. Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research. British. J. Nutr. 99:12-22.

Spigno, G., L. Tramelli, and D.M. De Faveri. 2007. Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. J. Food Eng. 81:200-208.

Sri Harsha, P.S., C. Gardana, P. Simonetti, G. Spigno, and V. Lavelli. 2013. Characterization of phenolics, in vitro reducing capacity and anti-glycation activity of red grape skins recovered from winemaking by-products. Bioresource Technol. 140:263-268.

Strube, M., L.O. Dragsted, and J.C. Larsen. 1993. Naturally occurring antitumourigens: plant phenols. Vol 1. Nordic council of ministers, CPH.

Sun, C.L., J.M. Yuan, M.J. Lee, C. S.Yang, Y.T. Gao, R. K.Ross, and M. C.Yu. 2002. Urinary tea polyphenols in relation to gastric and esophageal cancers: a prospective study of men in Shanghai, China. Carcinogenesis 3:1497–1503.

Thorngate, J. and V. Singleton. 1994. Localization of procyanidins in grape seeds. Amer. J. Enol. Viticult. 45:259-262.

Thunga, N. 2015. Finding value in grape pomace. MS Thesis, OK. State Univ., Stillwater

Torres, J.L., B. Varela, M.T. García, J. Carilla, C. Matito, J.J. Centelles, M. Cascante, X. Sort, and R. Bobet. 2002. Valorization of grape (*Vitis vinifera*) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. J. Agr. Food Chem. 50:7548-7555.

Treutter, D. 2006. Significance of flavonoids in plant resistance: a review. Environmental Chem. Lett. 4:147-157.

Tsao, R. 2010. Chemistry and biochemistry of dietary polyphenols. Nutrients 2:1231-1246.

Uchida, S. 1980. Condensed tannins scavenging active oxygen radicals. Med. Sci. Res. 15:831-832.

Vatai, T., M. Skerget, and Z. Knez. 2009. Extraction of phenolic compounds from elder berry and different grape marc varieties using organic solvents and/or supercritical carbon dioxide. J. Food Eng. 90:246-254.

Vidal, S., L. Francis, S. Guyot, N. Marnet, M. Kwiatkowski, R. Gawel, V. Cheynier, and E.J. Waters. 2003. The mouth- feel properties of grape and apple proanthocyanidins in a wine- like medium. J. Sci. Food Agr. 83:564-573.

Vilkhu, K., R. Mawson, L. Simons, and D. Bates. 2008. Applications and opportunities for ultrasound assisted extraction in the food industry — A review. Innovative Food Sci. Emerging Technol. 9:161-169.

Wang, L. and C.L. Weller. 2006. Recent advances in extraction of nutraceuticals from plants. Trends Food Sci. Technol. 17:300-312.

Waterhouse, A. 1999. Folin-Ciocalteau micro method for total phenol in wine. *no date), http://waterhouse. ucdavis. edu/phenol/folinmicro. htm (Accessed: May, 2005)*.

Yilmaz, Y. and R.T. Toledo. 2006. Oxygen radical absorbance capacities of grape/wine industry byproducts and effect of solvent type on extraction of grape seed polyphenols. J. Food Composition. Anal. 19:41-48.

Zhang, G., L. He, and M. Hu. 2011. Optimized ultrasonic-assisted extraction of flavonoids from *Prunella vulgaris* L. and evaluation of antioxidant activities in vitro. Innovative. Food Sci. Emerging Technol. 12:18-25.

VITA

GOVINDA SAPKOTA
Candidate for the Degree of

Master of Science

Thesis: OPTIMIZING PROANTHOCYANIDIN EXTRACTION FROM GRAPE SEEDS IN WINERY WASTES

Major Field: Horticulture

Biographical:

Education:

Completed the requirements for the Master of Science/Arts in your major at Oklahoma State University, Stillwater, Oklahoma in May, 2018.

Completed the requirements for the Bachelor of Science in Agriculture, Tribhuvan University/Kathmandu, Nepal in 2013.

Experience:

Employed by Oklahoma State University, Department of Horticulture and Landscape Architecture as a graduate research assistant under Dr. Niels Maness.

Professional Memberships: American Society for Horticultural Science (ASHS).