INFLUENCE OF SOLVENT EXTRACTION AND WINEMAKING STEPS ON ANTIOXIDANT ACTIVITY OF RED GRAPES

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OF RED GRAPES

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CHAPTER I

INTRODUCTION

Food processing is an industrial area that generates significant amounts of waste products, with fruit and vegetable industries being a major source of potentially valuable byproducts. Many research groups have shown promising results for the use of byproducts in food industry and human nutrition, and their utilization can increase the economic profitability of a crop, not only by the means of further processing, but also by decreasing the cost of disposal (Ruberto et al. 2008, Valiente et al. 1995).

Grapes are one of the largest cultivated fruit crops (ranking number #15 in the world in terms of commodity value), with 67 million tons produced in 2007 (according to information found on the web site http://faostat.fao.org). Grapes consequently generate large amounts of press residues, which originate mainly from wine production. About 13-20% of the total weight of grapes used in wine processing is lost as pomace (Brenes et al. 2008, Ruberto et al. 2008), which consists mainly of grape skins, seeds and stems (stalks).

The chemical composition of grape pomace consists mainly of compounds such as: alcohols, acids, aldehydes, esters, pectins, polyphenols, mineral substances and sugars (Ruberto et al. 2008). Grape skins and seeds contain flavonols and monomeric

phenolic compounds such as catechin, (-)-epicatechin, (-)-epicatechin-3-*O*-gallate, as well as dimeric, trimeric and tetrameric procyanidins (Brenes et al. 2008). Grape seeds are composed primarily of fiber (40%), oil (16%), protein (11%), and various phenolic compounds (7%). Grape skins are a good source of anthocyanins and anthocyanidins, while stems are high in tannins (de Campos et al. 2008).

Many beneficial health effects have been reported from the consumption of grapes and red wine (Llobera and Canellas, 2007), mainly due to the antioxidant and antimutagenic activity of the above bioactive components, especially in connection with cardiovascular diseases and cancer prevention (Leblanc et al. 2008, Su et al. 2006, Hung et al. 2000, Zhao et al. 1999).

Potential value of these waste streams derives from different waste components, therefore our goal was to develop a methodology for screening the grape waste for potential value extracts, which are high in antioxidant activity and can be used both as nutraceutical supplements and as functional food and feed ingredients. More specifically, the objectives of our study were to:

<u>Experiment I</u>

1. Develop a method for efficient preparation and fractionation of samples rich in antioxidant compounds from Cynthiana grape pomace.
2. Measure the antioxidant activity of the extracts using Oxygen Radical Absorbance Capacity (ORAC) assay.
3. Identify the major antioxidant compounds in the pomace extracts using High Performance Liquid Chromatography (HPLC).

Experiment II

1. Simulate wine production in a lab scale environment from Cabernet franc grapes.
2. Determine the antioxidant profile of Cabernet franc wine during the various steps of winemaking, using ORAC and HPLC.
3. Determine the antioxidant profile of Cabernet franc pomace using ORAC and HPLC, from extracts obtained with the developed method of Experiment I.

REFERENCES

Brenes, A., S.G. S Lyago-Ayerdy, I. Arija, F. Saura-Calixto, A. Viveros, I. Go li and C. Centeno. 2008. Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens. Poultry science. 87(2):307-316.

de Campos, L.M.A.S., S.R.S. Ferreira, R.C. Pedrosa and F.V. Leimann. 2008. Free radical scavenging of grape pomace extracts from cabernet sauvingnon (*vitis vinifera*). Bioresource technology. 99(17):8413-8420.

Hung, L.M., J.K. Chen, S.S. Huang, R.S. Lee and M.J. Su. 2000. Cardioprotective effect of resveratrol, a natural antioxidant derived from grapes. Cardiovasc Res. 47(3):549-55.

Leblanc, M.R., P.W. Wilson and C.E. Johnson. 2008. Influence of pressing method on juice stilbene content in Muscadine and Bunch grapes. Journal of food science an official publication of the Institute of Food Technologists. 73(4):h58-h62.

Llobera, A. and J. Canellas. 2007. Dietary fibre content and antioxidant activity of Manto Negro red grape (vitis vinifera): Pomace and stem. Food chemistry. 101:659-666.

Ruberto, G., A. Renda, V. Amico and C. Tringali. 2008. Volatile components of grape pomaces from different cultivars of Sicilian vitis vinifera l [electronic resource]. Bioresource technology. 99(2):260-268.

Su, H.C., L.M. Hung and J.K. Chen. 2006. Resveratrol, a red wine antioxidant, possesses an insulin-like effect in streptozotocin-induced diabetic rats. Am J Physiol Endocrinol Metab. 290(6):E1339-46.

Torres, J.L., B. Varela, M.T. Garcia, 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. Journal of agricultural and food chemistry. 50:7548-7555.

Valiente, C., E. Arrigoni, R.M. Esteban and R. Amado. 1995. Grape pomace as a potential food fiber. Journal of Food Science. 60:818-820.

Zhao, J., J. Wang, Y. Chen and R. Agarwal. 1999. Anti-tumor-promoting activity of a polyphenolic fraction isolated from grape seeds in the mouse skin two-stage initiation-promotion protocol and identification of procyanidin b5-3'-gallate as the most effective antioxidant constituent. Carcinogenesis. 20(9):1737-45.

CHAPTER II

REVIEW OF LITERATURE

Wine Polyphenols and Health

Polyphenols constitute the most abundant antioxidants in the diet and their total dietary intake can be much higher than that of all other classes of phytochemicals and identified dietary antioxidants (Scalbert et al. 2005). Their intake can reach up to 1g/d, 10 times higher than that of vitamin C and 100 times higher than vitamin E and carotenoids (Manach et al. 2004). There are many dietary sources for polyphenols, mainly fruits, vegetables and plant-derived beverages (fruit juices, tea, coffee, and red wine).

The observed health and nutritional benefits of wine are mostly associated with the consumption of red wine, and as public awareness increases, so does public recognition of the term ‘French Paradox’, which describes the apparent conundrum whereby the incidence of coronary heart disease of French people is relatively low, even though their diet is relatively rich in saturated fats (German and Walzem, 2000; Cooper et al., 2004; Lindberg et al., 2008).

The nutritional and health benefits of wine have been associated with both ethanol and non-alcoholic compounds (German and Walzem, 2000) and there is also evidence that alcohol has a positive synergistic effect with wine polyphenols against atherosclerotic diseases (Cooper et al., 2004). However, wine health benefits are still not clearly defined and it is debatable whether the benefits are a result of wine's compounds or an effect of socioeconomic confounders such as income level and access to health care (Lindberg et al., 2008).

Alcohol concentration in typical table wines ranges 8-15%. Studies have associated moderate ethanol intakes, defined as 2-5 glasses of wine per day, with reduced mortality compared to abstinence from ethanol. However, consumption of wine above the moderate intake limits significantly increases mortality (MacDonald, 1999; Renaud et al., 1998). Moderate consumption of ethanol causes elevations in plasma high density lipoprotein (HDL) cholesterol that possesses protective properties against atherosclerotic cardiovascular disease (ASCVD).

Non-alcoholic compounds like phenolic acids and polyphenols are the major compounds in wine that possess antioxidant properties and may provide health benefits against cardiovascular diseases (German and Walzem, 2000; Cooper et al., 2004). Resveratrol is one phenolic compound that has been reported to have numerous health benefits including cardiovascular protective, anti-cancer and anti-inflammatory properties (Hung et al., 2000; Forester and Waterhouse, 2009). Resveratrol and other phenolic compounds may reduce the tendency of low density lipoproteins to become oxidized and participate in atherosclerosis (Anli et al. 2006, Cooper et al. 2004). Besides ASCVD,

cancer is another disease commonly associated with direct oxidative damage. Consequently, the antioxidant properties of wine are also believed to protect against several types, especially with epithelial cancers of alimentary and respiratory tracts (Su et al. 2006). Polyphenols could protect against cancer initiation by scavenging mutagens and carcinogens, and by shielding sensitive structures like DNA.

A phenolic compound consists of one or more hydroxyl groups attached directly to an aromatic or benzene ring. The antioxidant properties of phenolic compounds are mainly due to the conjugated bonds present in the benzene ring (Vermerris and Nicholson, 2006). When a free radical reacts with a phenolic, the radical is constantly delocalized between the conjugated bonds and thus not able to react with other molecules. There is a wide range of phenolic acids and polyphenols in wine, and each one may have individual biological effects, which make health effects of wine more complicated (Shahidi and Naczk, 2003). The two main groups of polyphenols are flavonoids (anthocyanins, flavan-3-ol monomers and polymers, flavonols, dihydroflavonols) and non-flavonoids (hydrobenzoic acid and hydroxycinnamic acid and their derivatives, stilbenes and phenolic alcohols) (German and Walzem, 2000). Polyphenols in red wines suppress the synthesis of the peptide endothelin-1 associated with vasoconstricting effects (Corder et al., 2006). They are also able to chelate transition metals.

Phenolic compounds are sensitive to oxidation, light and heat. Therefore the best conditions to maintain their activity, is to minimize exposure to these degradation factors. For example, storage humidity for bottled wine should be around 60-70%; not too dry because air percolates through the cork, nor too high because mildew or rot might

develop on the cork. Wines should be stored in a dark place to avoid light exposure, and normally in tinted dark colored glass bottles, either horizontally or at 45^{o} angle towards the bottom, to keep the cork moist enough and prevent air penetration. As in most storage conditions, the best preventive mechanism is correct temperature management. The optimal temperature for wines is 10-13^{o}C and minimal temperature fluctuation is preferred, again to avoid oxygen permeation (according to information found on the web site of Basic Wine Knowledge, 2009).

Extraction methods for phenolic compounds

A lot of research has been done on extraction of phenolic compounds from different food matrices using a variety of solvents and procedures. A summary of several studies and their major results follows.

Duddonė et al. (2009), extracted **aqueous** fractions from 30 plant material **for total phenolic content** and antioxidant capacity. They found that oak (*Quercus robur*), pine (*Pinus maritima*), and cinnamon (*Cinnamomum zeylanicum*) extracts showed the highest phenolic content (300-400 mg GAE/g) as well as the highest antioxidant capacities as measured by several methods (DPPH and ABTS radical scavenging capacity assay, ORAC assay, superoxide dismutase (SOD) assay, and ferric reducing antioxidant potential (FRAP) assay. Thus, a strong, positive correlation between phenolic content and antioxidant activity was generally observed, suggesting that phenolic compounds are responsible for the antioxidant properties of these plants extracts. They extracted phenolics by agitating ground tissue (125g) with distilled water at 50°C. Extracts were filtered and water was removed in a rotary evaporator at 50°C to obtain a

powder, which was used for subsequent analyses (See appendix, pp. 86-87 for select results).

Bellido and Beta (2009) looked for **anthocyanin content** and antioxidant activity, between a bran-rich pearling fraction (10% outer kernel layers) and whole kernel flour obtained from different cereal grain genotypes of barley. They found that the anthocyanin content of the bran-rich fractions of yellow and purple barley was up to 6 times more per unit weight (1587 and 3534μg/g, respectively) than their corresponding whole kernel flours (210 and 573, respectively). Anthocyanins were extracted as follows: barley samples (2.5g) were shaken for 2.5h (at 25°C) in 25mL of acidified methanol (1N HCL, 85:15, v/v) after pH adjustment to 1.0 (with 1N HCl). The extract was sonicated for 30min (at 25°C) and centrifuged for another 30min at 10000rpm (at 15°C). The supernatant was filtered (0.45μm nylon syringe filter), and concentrated x10 under nitrogen at 40°C. The final pH was readjusted to 1.0 (25°C) (See appendix, p. 88 for select figures & tables).

The group of Ross et al. (2009) used different hydrolysis methods and High Performance Liquid Chromatography (HPLC) to determine the **phenolic acid content** of three different varieties of dry beans. They tested three different methods, a sequential hydrolysis of base and acid extraction, a pure base and a pure acid hydrolysis. They also examined the protective effect of Ascorbic acid (AA) and Ethylene-diamine-tetraacetic acid (EDTA). They observed that the majority of phenolic acids were extracted from the base hydrolyzed fraction of the sequential hydrolysis method. They also observed that AA and EDTA exhibited a protective effect when added to the mix. Acid hydrolysis released some additional compounds present in the beans that were not detected with

base hydrolysis. For the free phenolic acid extraction from 0.5g tissue they used 7mL acidified methanol (85:15; v/v methanol/10% acetic acid), sonicated for 30 min. Volume was adjusted to 10mL with distilled water, and 1mL was filtered through a 0.45μm Polytetrafluoroethylene (PTFE) filter for free phenolic acid determination with HPLC. The remaining 9mL were used for the sequential base/acid extraction. The pure base and pure acid extraction methods followed a different procedure.

Anthocyanin content of grape juices from different grape varieties grown in Korea was studied by Oh et al. (2008). They found that the predominant anthocyanins where different depending on cultivar, or that even when the same compounds where identified, the proportions were different in different cultivars. They also noticed that HPLC column temperature had an effect on peak separation and suggested 35^{o}C for their purposes (Appendix, p. 89). They extracted anthocyanins by first rinsing the sample (15mL of juice) from sugars and other water-soluble components using double distilled water (60mL) on an open glass column packed with Amberlite XAD-7 (0.8cm × 16cm). The anthocyanins were obtained by elution with 30mL of 0.1% HCL in methanol. Extracts were filtered and concentrated when appropriate for HPLC analysis.

Guerrero et al. (2008) tested the extraction of **total polyphenols** from distilled white grape pomace using a lab-scale vertical extractor and water or ethanol as extraction solvent. They also monitored the influence of flow (2 mL/min and 4 mL/min) and temperature (40^{o}C and 50^{o}C) on the extraction yields. They found that the aqueous extracts contained up to 60 times more polyphenols than the ethanolic extracts (Appendix, p. 90), which they mention was in contrast with some previous work they had done on batch extraction from different grape varieties. The extraction of polyphenols

from ground grape pomace (27g dry weight) was performed in a laboratory extractor (4.5cm in.diam., 10cm height), at constant temperature in water bath. A pump was attached to the bottom and a condenser to the top of the extractor to provide constant solvent flow and prevent solvent loss respectively.

The biological activity of **phenolic extracts** from red wine and pomace against *Streptococcus mutans* was investigated by Thimothe et al. (2007). They found variable anthocyanin and flavan-3-ols content due to grape variety and extraction source (whole fruit versus pomace) and made suggestions regarding the effectiveness of grape phenolic extracts, especially from pomace, against specific virulence traits of *S. mutans*. They extracted polyphenols from freeze-dried samples (20g), using 200mL of methanol/ethanol/water (50/25/25%, v/v) for 20min in a sonicator on ice. Samples were centrifuged at 10000 rpm for 20min and the procedure was repeated. After solvent evaporation at 35°C, the aqueous polyphenolic extract was passed through preconditioned C18 Sep Pak cartridges, washed with 0.01N aqueous HCl and dried under nitrogen. Polyphenols were collected with elution of methanol, and after solvent evaporation, were re-suspended in distilled water, lyophilized (powder form) and re-solubilized in methanol. After acid hydrolysis with 2M HCl by heating for 1h at 90°C samples were injected to HPLC for analysis (peak separation included in appendix, p. 93).

In a study of Ruberto et al. (2007), the polyphenol content and the antioxidant activity of 5 different red grape cultivars was examined. They targeted **anthocyanins and flavonols**, which they identified with High Performance Liquid Chromatography Ultra Violet Diode Array Detector (HPLC–UV–DAD) and High Performance Liquid

Chromatography Mass Spectra Electron Spray Ionization (HPLC–MS–ESI). They observed a large degree of variability in the total contents of anthocyanin and flavonol in their extracts as well as poor correlation with antioxidant activity. For the collection of anthocyanins and flavonols they extracted 50g of sample (freeze-dried and ground) 3 times with 300mL of 1% 1N HCl at 25^{o}C for 4 h with continuous stirring. The combined solution was concentrated to 300mL and extracted another 3 times with 300mL of hexane. Both organic layers were evaporated and the methanolic extract was used for analyses.

Pomace samples from red and white grapes were evaluated by Kammerer et al. (2004) for **phenolic compounds.** Using HPLC-MS and HPLC-DAD they were able to identify and quantify anthocyanins, hydroxybenzoic and hydroxycinnamic acids, catechins, flavonols and stilbenes in the skins and seeds. Cultivar and vintage showed an effect on phenolic compound composition; grape skins were rich in anthocyanins, hydroxycinnamic acids, flavanols, and flavonol glycosides, while seeds were rich in flavonols. The only difference between red and white pomace samples was the absence of anthocyanins in white grape pomace. For extraction of phenolic compounds they used different solvents and fractions, depending on the phenolics of interest, during a stepwise procedure. They separated seeds and skins which were lyophilized and ground. Anthocyanins were extracted twice (1:20 sample:solvent for 2h and 15min respectively) with methanol/0.1% HCl (v/v), supernatants were combined, evaporated to dryness at 30°C, and re-dissolved in 20mL of acidified water (pH 3.0). Extracts were directly injected for analysis. For non-anthocyanin phenolics in red grape skin, 5mL of the skin extracts were made up to 20mL; pH was adjusted to 1.5 and extracted 4x50mL ethyl

acetate. Extracts were combined, evaporated to dryness, dissolved in water, adjusted the pH to 7.0 and applied to preconditioned solid phase extraction (SPE) cartridges. All other extracts (5mL each) were adjusted to pH 7.0 and applied directly to the preconditioned cartridges. For recovery of phenolic acids SPE cartridges were rinsed with 10mL DI water and 10mL 0.01% HCl, concentrated under vacuum and re-dissolved in 2% acetic acid; for anthoxanthins and stilbenes SPE cartridges were eluted with 20mL of ethyl acetate, concentrated under vacuum and re-dissolved in methanol. All samples were membrane-filtered (0.45μm) before HPLC injection (separation included in appendix, pp. 91-92) .

Antioxidant activity and Oxygen Radical Absorbance Assay (ORAC)

There are several assays for testing antioxidant activity in food products. A summary of a few common tests (Seeram et al. 2008) includes:

1. Trolox Equivalent Antioxidative Capacity (TEAC). This test uses ABTS radical cations and Trolox as a standard. It requires a microplate reader. Samples are mixed in a Na/K buffer with ABTS radical solution and absorbance is obtained after 5min at 750nm. Results are compared with a Trolox standard curve and expressed as Trolox equivalents.
2. Oxygen Radical Absorbance Capacity (ORAC). This assay normally uses a fluorescent probe (*β*-phycoerythrin or fluorescein) and a free radical generator (e.g. AAPH). Trolox is also used as a standard. Degradation of the fluorescent probe is monitored over time (about 1h), typically using a microplate reader. Sample degradation curves are compared against Trolox and a blank

(phosphate buffer) degradation curve (areas under the curve) and results are also reported as Trolox equivalents.

3. Ferric Reducing Antioxidant Power (FRAP). TPTZ (2,4,6-tri[2-pyridyl-s-triazine]), ferric chloride and sodium acetate buffer are mixed at 1:1:10 ratio and a portion is heated at 37^{o}C for 10min. Aqueous sample extracts are added and absorbance is measured at 593nm using a microplate reader. Results are compared with ferrous sulfate standards and using linear regression, are expressed in mmolar ferric ions converted to the ferrous form/mL.
4. Free radical Scavenging using DPPH radical. DPPH is a radical generator and has a deep violet color due to its unpaired electron. An ethanolic solution of DPPH is mixed with the sample for analysis and change in their optical density is monitored at 517nm using a microplate reader.

These methods are very commonly used for several food matrices, including phenolic compounds in wines and grape pomace. The ORAC assay has been widely used and undergone through several improvement steps from research groups, and is considered a reliable method for comparison of antioxidant capacity of compounds from different food matrices.

One of the major components of assays such as ORAC, is the fluorescent probe used as target for radical attack. Parinaric acid (-cis) has been used in the past as a probe for lipid peroxidation (Kuypers et al. 1987), however a major disadvantage of this indicator was that it had to be excited at UV 320nm, where most test samples absorbed (Naguib 2000). In addition, it is air and photo-sensitive, resulting in loss of fluorescence. Extensive use of β-phycoerythrin has been employed by researchers as a target molecule

of free radical attack for the ORAC assay. Ehlenfeldt and Prior (2001) used it to assess phenolic and anthocyanin concentrations in fruit and leaf tissues of Highbush blueberry. Antioxidant capacity of different broccoli (*Brassica oleracea*) genotypes was investigated by Kurilich et al. (2002), while Cao and Prior (1999) examined the antioxidant capacities of several biological samples (plasma, serum, wine, fruits, vegetables, and animal tissues) using the same probe. Naguib (2000) tested an alternative indicator (6-carboxyfluoroscein) on water soluble antioxidants (Trolox, ascorbic acid, uric acid, quercetin, and rutin), in serum samples and showed a linear correlation of the net protection value with the concentration of serum, Trolox, ascorbic acid, and uric acid.

In addition, Ou et al. (2001) demonstrated that *β*-phycoerythrin has several disadvantages, such as variable reactivity with peroxyl radical which results in inconsistency, 'photobleaching' with exposure to excitation light, and potential interaction with polyphenols in samples (also reported by Naguib 2000). They developed and validated the use of fluorescein (FL) (3',6'-dihydroxyspiro[isobenzofuran-1[3*H*],9'[9*H*]-xanthen]-3-one) as the fluorescent probe, showing more consistent results than with *β*-phycoerythrin and suggested that the ORACFL assay can provide a reliable, direct measurement of antioxidants that protect against the peroxyl radical's ability to break hydrophyllic chains. Since its first use, fluorescein has been adapted from many research groups as a fluorescent agent on antioxidant assays with ORAC.

Huang et al. (2002a) further investigated the use of fluorescein as the fluorescent probe for ORAC assays. They studied the applicability of ORAC for testing lipophilic antioxidants, since the major use of ORAC had been limited to hydrophilic antioxidants due to the aqueous nature of the assay. They employed randomly methylated *β*-

cyclodextrin (RMCD) as a water solubility enhancer and found that at 7% in 50% acetone-water, RMCD was able to solubilize vitamin E and other lipophilic compounds in 75mM phosphate buffer (pH 7.4).

The latter group of researchers (Huang et al. 2002b) presented an automated instrument platform for the ORAC assay procedure to reduce sample preparation and labor. They used an eight-channel robotic liquid handling system and a microplate fluorescence reader. They showed a 10-fold increase on the efficiency of the assay with low detection and quantification limits (5 and 6.25 μM respectively).

HPLC settings for phenolic compound determination

High Performance Liquid Chromatography has been widely employed, either alone or coupled to a Mass Spectrophotometer, to identify and quantify polyphenolic extracts. A plethora of mobile phase combinations, gradient styles and micro settings are described in the literature, and in this section we try to present several procedures that may help explain the design of our identification protocol.

Amico et al. (2008) identified the **anthocyanin and flavonol/flavonol glycosides** in polyphenol-enriched fractions of grape pomace of red wines. They identified the following anthocyanins: Delphinidin 3-*O*-glucoside, Cyanidin 3-*O*-glucoside, Petunidin 3-*O*-glucoside, Peonidin 3-*O*-glucoside, Malvidin 3-*O*-glucoside, Malvidin 3-*O*-(6”-*O*-p-coumaroyl)-glucoside, and flavonol/flavonol glycosides: Myricetin 3-*O*-glucoside, Quercetin 3-*O*-glucuronide, Quercetin 3-*O*-glucoside, Isorhamnetin 3-*O*-glucuronide and Quercetin. For determination they used HPLC–DAD with a reverse phase column and mobile phases of solvent A: Water-Acetic Acid, 9:1 (v/v); and solvent B: Acetonitrile-

Acetic Acid, 9:1 (v/v) at a gradient of t0 min 5% B, t20 min 15% B, t40 min 30% B, t55 min 100% B, and t65 min 100% B. The flow rate was 1mL/min and detection was set between 200 and 700 nm, were anthocyanins were detected at 530 nm and the flavonols and flavonol glycosides were detected at 350 nm. Calibration curves were constructed using standards of malvidin 3-*O*-glucoside chloride and quercetin 3-*O*-glucoside.

Thimothe et al. (2007) identified **phenolic extracts** from red wine and pomace whose biological activity was tested against *Streptococcus mutans*. They used a reversed-phase C18 Symmetry Analytical column (5µm × 250mm × 4.6mm) with a Symmetry Sentry guard column on a Hewlett-Packard HPLC system, model 1100. For separation their mobile included: Solvent A) 0.1% phosphoric acid (H3PO4) in HPLC-grade water and Solvent B) 0.1% H3PO4 in HPLC-grade acetonitrile with a gradient of: t0=8% B, t4= 11% B, t25= 35% B, t30=60% B, t40=60% B, t45=35% B, t50= 11% B, t55= 8% B for a total of 55min. Flow rate was at 1mL/min, with detector set to 280, 320, 370, and 520 nm. Calibration curves were used for the following standards: gallic acid, caffeic acid, ferulic acid, p-coumaric acid, sinapic acid, shikimic acid, chlorogenic acid, catechin, epicatechin, procyanidin B1, procyanidin B2, resveratrol, myricetin, quercetin, kaempferol, isorhamnetin, naringin, delphinidin, cyanidin, petunidin, peonidin, and malvidin.

Ruberto et al. (2007), studied the polyphenol content and the antioxidant activity of 5 different red grape cultivars, and used HPLC to identify **anthocyanins and flavonols**. They used a Phenomenex Luna C18 column (250 x 4.6 mm, 5µm) at 20^{o}C with mobile phases of Solvent A) water:formic acid, 9:1 (v/v) and Solvent B) acetonitrile:formic acid, 9:1 (v/v). The gradient was set as follows: t0= 5% B, t20= 15%

B, t40= 30% B, t55= 100% B, t65=100% B. Flow rate was 1mL/min and the detector was set at a range between 200 and 700nm, with 350nm being for flavonols and flavonol glycosides, 480nm for pyranoanthocyanins, and 530nm for anthocyanins (Table of anthocyanin concentrations included in appendix, p. 94).

Kammerer et al. (2004) used HPLC-MS and HPLC-DAD to identify and quantify **phenolic compounds** from red and white grape pomace**.** They used three different settings for each group of interest; 1) for anthocyanins, 2) for phenolic acids and 3) for anthoxanthins and stilbenes. Their HPLC system was an Agilent HPLC series 1100, equipped with a degasser, a binary gradient pump, a thermo-autosampler, a column oven, and a diode array detector. They used a Phenomenex Aqua C18 column (250 x 4.6mm i.d.; 5µm particle size) and a C18 ODS guard column (4.0 x 3.0mm i.d.), at 25 °C. Spectral range was set at 200-600nm at 1.25 scans/s (peak width = 0.2 min). For **anthocyanin** separation their mobile phase was: Solvent A) water/formic acid/acetonitrile (87:10:3, v/v/v) and Solvent B) water/formic acid/acetonitrile (40:10:50, v/v/v) with a gradient of: t0= 10% B, t10= 25% B, t15= 31% B, t20= 40% B, t30= 50% B, t40= 100%B, t45= 10% B for total run time of 50min. Detection was set at 520nm at a flow rate of 0.8mL/min and 1-25µL injection. For **phenolic acids** separation, the mobile phase was: Solvent A) 2% (v/v) acetic acid in water and Solvent B) 0.5% acetic acid in water and acetonitrile (50:50, v/v) with a gradient of: t0= 10% B, t10= 15% B, t13= 15% B (isocratic), t20= 25% B, t50= 55% B, t51= 100% B, t56= 100% B (isocratic), t=56.1 10% B for a total run time of 60min. Detection was set at 280nm (hydroxybenzoic acids) and 320nm (hydroxycinnamic acids) at a flow rate of 1.0mL/min and 5-10µL injection. For **anthoxanthins and stilbenes** separation, the mobile phase was: Solvent A) 2% (v/v)

acetic acid in water and Solvent B) 0.5% acetic acid in water and acetonitrile (50:50, v/v) with a gradient of: t0=10% B, t20= 24% B, t40= 30% B, t60= 55% B, t75= 100% B, t83= 100% B (isocratic), t85= 10% B for a total run time of 90min. Detection was set at 280nm (flavanols), 320nm (stilbenes), and at 370nm (flavonols) at a flow rate of 1.0mL/min and 10μL injection (Figures & tables included in appendix, pp. 91-92).

Summary

It is apparent that polyphenolic compounds have several beneficial effects towards health and are considered one of the major natural sources of antioxidants. A plethora of research has demonstrated the value of these compounds and a variety of procedures have been employed to extract them out of their natural matrix. Grapes, grape products and byproducts have been also well investigated and found to contain significant amounts of phenolic compounds. Winery waste streams are potential sources of antioxidants and a methodology of bulk industrial extraction would be beneficial in utilizing and concentrating the compounds of interest. Our project compared the efficiency of different single solvents in extracting antioxidants from grape pomace that would also have a scale up potential for industrial application.

REFERENCES

Amico, V., R. Chillemi, S. Mangiafico, C. Spatafora and C. Tringali. 2008. Polyphenol-enriched fractions from Sicilian grape pomace: HPLC-DAD analysis and antioxidant activity. Bioresource technology. 99 (13):5960-5966.

Anli, E., N. Vural, S. Demiray and M. Ozkan. 2006. Trans-resveratrol and Other Phenolic Compounds in Turkish Red Wines with HPLC. Journal of Wine Research. 17 (2):9p.

Basic Wine Knowledge – Proper Wine Storage. 2009. www.basic-wine-knowledge.com/proper-wine-storage.html (17 November 2009).

Bellido, G.G. and T. Beta. 2009. Anthocyanin Composition and Oxygen Radical Scavenging Capacity (ORAC) of Milled and Pearled Purple, Black, and Common Barley. Journal of agricultural and food chemistry. 57 (3):1022-1028.

Cao, G. and R.L. Prior. 1999. Measurement of oxygen radical absorbance capacity in biological samples. Methods Enzymol. 299 50-62.

Cooper, K.A., M. Chopra and D.I. Thurnham. 2004. Wine polyphenols and promotion of cardiac health. Nutr Res Rev. 17 (1):111-30.

Dudonne, S., M. Woillez, J.-M. Merillon, X. Vitrac and P. Coutiere. 2009. Comparative Study of Antioxidant Properties and Total Phenolic Content of 30 Plant Extracts of Industrial Interest Using DPPH, ABTS, FRAP, SOD, and ORAC Assays. Journal of agricultural and food chemistry. 57 (5):1768-1774.

Ehlenfeldt, M.K. and R.L. Prior. 2001. Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry. Journal of agricultural and food chemistry. 49 2222-2227.

German, J.B. and R.L. Walzem. 2000. The health benefits of wine. Annual review of nutrition:561-593.

Guerrero, M.S., J.S. Torres and M.J. Nunez. 2008. Extraction of polyphenols from white distilled grape pomace: Optimization and modelling. Bioresource technology. 99 (5):1311-1318.

Huang, D., B. Ou, M. Hampsch-Woodill, J.A. Flanagan and E.K. Deemer. 2002a. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated beta-cyclodextrin as the solubility enhancer. Journal of agricultural and food chemistry. 50 1815-1821.

Huang, D., B. Ou, M. Hampsch-Woodill, J.A. Flanagan and R.L. Prior. 2002b. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. Journal of agricultural and food chemistry. 50 4437-4444.

Kammerer, D., A. Claus, R. Carle and A. Schieber. 2004. Polyphenol screening of pomace from red and white grape varieties (*Vitis vinifera* L.) by HPLC-DAD-MS/MS. Journal of agricultural and food chemistry. 52 (14):4360-4367.

Kurilich, A.C., E.H. Jeffery, J.A. Juvik, M.A. Wallig and B.P. Klein. 2002. Antioxidant capacity of different broccoli (Brassica oleracea) genotypes using the oxygen

radical absorbance capacity (ORAC) assay. Journal of agricultural and food chemistry. 50 5053-5057.

Kuypers, F.A., J.J. van den Berg, C. Schalkwijk, B. Roelofsen and J.A. Op den Kamp. 1987. Parinaric acid as a sensitive fluorescent probe for the determination of lipid peroxidation. Biochim Biophys Acta. 921 (2):266-74.

MacDonald I. 1999. Health Issues Related to Alcohol Consumption. Oxford, UK: Blackwell Sci. 488 pp. 2nd ed.

Manach, C., A. Scalbert, C. Morand, C. Remesy and L. Jimenez. 2004. Polyphenols: Food sources and bioavailability. Am J Clin Nutr. 79 (5):727-47.

Naguib, Y.M. 2000. A fluorometric method for measurement of oxygen radical-scavenging activity of water-soluble antioxidants. Anal Biochem. 284 (1):93-8.

Oh, Y.S., D.S. Choi, E. Choe, M.Y. Jung, J.H. Lee, S.H. Yoon and C.H. Oh. 2008. Characterization and Quantification of Anthocyanins in Grape Juices Obtained from the Grapes Cultivated in Korea by HPLC/DAD, HPLC/MS, and HPLC/MS/MS. Journal of food science an official publication of the Institute of Food Technologists. 73 (5):c378-c389.

Ou, B., R.L. Prior and M. Hampsch-Woodill. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. Journal of agricultural and food chemistry. 49 4169-4626.

Renaud, S.C., R. Gueguen, J. Schenker and A. d'Houtaud. 1998. Alcohol and mortality in middle-aged men from eastern France. Epidemiology. 9 (2):184-188.

Ross, K.A., S.D. Arntfield and T. Beta. 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 chemistry. 113 (1):336-344.

Ruberto, G., A. Renda, C. Daquino, V. Amico, C. Spatafora, C. Tringali and N. De Tommasi. 2007. Polyphenol constituents and antioxidant activity of grape pomace extracts from five Sicilian red grape cultivars. Food chemistry. 100: 203-210.

Seeram, N.P., L. Feng, M. Dreher, D. Heber, M. Aviram, Y. Zhang and S.M. Henning. 2008. Comparison of Antioxidant Potency of Commonly Consumed Polyphenol-Rich Beverages in the United States. Journal of agricultural and food chemistry. 56 (4):1415-1422.

Shahidi, F. and M. Naczk. 2000. Phenolics in food and nutraceuticals / Fereidoon Shahidi and Marian Naczk. Boca Raton, Fla.: CRC Press.

Scalbert, A., I.T. Johnson and M. Saltmarsh. 2005. Polyphenols: Antioxidants and beyond. Am J Clin Nutr. 81(1):215S-217S.

Su, H.C., L.M. Hung and J.K. Chen. 2006. Resveratrol, a red wine antioxidant, possesses an insulin-like effect in streptozotocin-induced diabetic rats. Am J Physiol Endocrinol Metab. 290 (6):E1339-46.

Thimothe, J., I.A. Bonsi, O.I. Padilla-Zakour and H. Koo. 2007. Chemical Characterization of Red Wine Grape (*Vitis vinifera* and *Vitis* Interspecific Hybrids) and Pomace Phenolic Extracts and Their Biological Activity against *Streptococcus mutans*. Journal of agricultural and food chemistry. 55 (25):10200-10207.

Watkins, T.R., American Chemical Society. Division of Agricultural and Food Chemistry. and American Chemical Society. Meeting. 1997. Wine : nutritional and therapeutic benefits / Tom R. Watkins, editor. Washington, DC: American Chemical Society.

Vermerris, W. and R. Nicholson. 2006. Phenolic compound biochemistry / by Wilfred Vermerris and Ralph Nicholson. Dordrecht: Springer.

CHAPTER III

Experiment I

Influence of solvent extraction methods on antioxidant activity of Cynthiana grape pomace extracts. Quantitative and qualitative analysis by ORAC and HPLC.

ABSTRACT

As interest in dietary antioxidants have increased in recent years, researchers have investigated using horticultural processing industry waste streams as feedstocks for obtaining these valuable by-products. Our study focused on developing a rapid and scalable method for screening processing waste streams for antioxidant activity using different combinations of solvents; winery waste was used as a model. Pressed grape pomace from the cultivar Cynthiana (*Vitis aestivalis*) was screened, frozen in liquid nitrogen, and ground. Powder was sieved and samples were extracted using four solvents: 100% petroleum ether (PE), 70% methanol (MT), 50% acetone (AC), and 0.01% pectinase (PC) in water. These were used at 2:1 and 4:1 solvent:pomace ratios. Samples were extracted for 1, 2, 4 and 8 hours. Extracts were then filtered and stored at -20°C. Antioxidant activity was quantified using the Oxygen Radical Absorbance Capacity (ORAC) assay and expressed as μmoles of Trolox equivalents/g of grape pomace. HPLC

analysis was performed to identify the major polyphenolic compounds in Cynthiana pomace. Our results showed that all extracts had antioxidant activity, which was influenced by the choice of solvent. Extraction efficiency for antioxidants was significantly different ($p<0.05$) among all solvents. Higher activity was observed in the 50% Acetone and 70% Methanol extracts, with average of 32 and 21 μmoles Trolox equivalents/g pomace respectively. Acetone extracts showed significantly ($p<0.05$) higher activity than methanol extracts in both solvent ratios and all extraction times. Activity ranged from 27-37μmoles Trolox equivalents/g pomace for acetone and 18-24μmoles Trolox equivalents/g pomace for methanol. The antioxidant activities of petroleum ether and pectinase/water extracts were much lower, at 2 and 5 μmoles Trolox equivalents/g pomace respectively. For all solvents, 2h of extraction at 4:1 solvent to sample ratio showed the highest activity, except for petroleum ether where ratio showed no difference. HPLC analysis of our best extraction method showed that cyanidin 3-*O*-glucoside, malvidin 3-*O*-glucoside, epicatechin and coumaric acid were the most prevalent compounds in the extracts, while less amounts of caffeic acid, quercetin and catechin were also detected. Several major peaks were not identified with our set of standards, suggesting that other compounds are present in significant amounts in Cynthiana pomace, compounds that may merit further investigation. Overall, our screening method allowed us to identify potentially high-value grape processing waste products, thus paving the way toward developing a commercially-viable method for extracting antioxidants from grape pomace.

INTRODUCTION

Fruit and vegetable industries generate significant amounts of waste products, which may be a major source of potentially valuable byproducts. Their utilization can increase the economic profitability of a crop, not only by the means of further processing, but also by decreasing the cost of disposal (Ruberto et al. 2008, Valiente et al. 1995).

Grapes, being one of the largest cultivated fruit crops with 67 million tons produced in 2007 (Food and Agriculture Organization of the United Nations, 2009), generate large amounts of press residues, which originate mainly from wine production. About 13-20% is lost as pomace (Brenes et al. 2008, Ruberto et al. 2008), which consists mainly of grape skins, seeds and stems (stalks).

Many beneficial health effects have been reported from the consumption of grapes and red wine (Llobera and Canellas, 2007), mainly due to the antioxidant and antimutagenic activity of the above bioactive components, especially in connection with cardiovascular diseases and cancer prevention (Leblanc et al. 2008, Su et al. 2006, Hung et al. 2000, Zhao et al. 1999).

Since polyphenolic compounds are linked with these antioxidant properties and health benefits, a great interest has emerged in bulk extraction of these compounds from such waste streams. A lot of research has been done on extraction methods for polyphenolic antioxidants from grapes and grape pomace, but most of the times a combination of solvents and/or several tedious steps are involved for their recovery, which would have limited industrial applicability.

Our goal was to compare the use of different single solvent mixtures on their efficiency to extract these compounds from grape pomace, and still have a potentially

practical application in a bulk scale. Grape pomace of the cultivar Cynthiana (*vitis aestivalis*) was used to compare solvent efficiency on antioxidant extraction, which was quantified using the ORAC assay. Identification of the major antioxidant components was determined by HPLC.

MATERIALS AND METHODS

Chemicals

ORAC: Potasium phosphate dibasic (K_2HPO_4) ACS (EM Science, US)), sodium phosphate monobasic ($NaHPO_4*H_2O$) ACS (Fisher Scientific NJ, US), AAPH [2,2'-azobis(2-amidino-propane) dihydrochloride] (Waco Chemicals Inc., Richmond, VA), Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Fluka Chemika, Switcherland), fluorescein disodium (Sigma-Aldrich, St-Louis, MO), Randomly Methylated *β*-Cyclodextrin (RMCD) (Cyclodextrin Technologies Development Inc., www.cyclodex.com).

HPLC: HPLC grade solvents and analytical reagent grade ortho-phosphoric acid were purchased from Fisher Scientific (Fair Lawn, NJ). Deionized water was produced by a Milli-Q unit (Millipore, Bedford, MA, USA). Gallic acid, catechin monohydrate, epicatechin, caffeic acid, coumaric acid, ferrulic acid, myricetin, quercetin, trans-resveratrol, kaempferol and isorhamnetin were purchased from Fisher Scientific (Fair Lawn, NJ); cyanidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside were obtained from Polyphenols Laboratories AS (Sandnes, Norway).

Miscellaneous: Folin-Ciocalteu reagent (Fluka BioChemica, Switzerland), gallic acid and sodium carbonate (Sigma Aldrich, St. Louis, MO).

Sample Collection and Preparation

Pomace samples were obtained from a local winery and consisted of pressed Cynthiana var. 'Norton' grapes. Approximately two full 55gal barrels were acquired and transferred in laboratory facilities in Stillwater, OK. Upon arrival, samples were immediately vacuum sealed (Multivac C500, Multivac Inc. Kansas City, MO) in individual clear plastic bags (8 x 14 inches) of approximately 3lb each and distributed in 8 plastic totes at -20^{o}C until further processing.

Four plastic bags from each tote were randomly selected and allowed to thaw at refrigeration temperature (2-4^{o}C) overnight. Each bag was individually checked for wood chips, stones and large woody stem residues from the winery process. Contents were combined in a large container and thoroughly mixed. Approximately 4.0kg (1/10th of the selected total) of the mixed pomace was randomly selected for sample preparation.

Combined pomace mixture was transferred in cold room (≈4^{o}C) and rapidly frozen in liquid nitrogen using a metal kitchen strainer. At same conditions, samples were immediately ground into a powder using a commercial Warring blender (model 51BL31) and stored in 1 gallon freezer Ziploc bags. Ground tissue was sieved using a Tyler equivalent #6 standard sieve (W.S. Tyler, US) and stored in a freezer at -20^{o}C until sample extraction.

Composition analysis

Representative samples of pomace were collected separately for moisture content, pH and water activity. Moisture content was performed for raw grape pomace as well as for several treatments of freeze dried samples that were lyophilized for 24, 48, 72 and 96h. For this purpose, pomace was manually separated into skins and seeds and duplicate 2g samples were dried at 80^{O}C under vacuum (15in. Hg) until constant weight was observed. Water activity and pH were obtained using an AquaLab Series 3 (Decagon Devices, Inc., Pullman WA) water activity meter and an Accumet pH meter (AB15 Basic, Fisher Scientific, Denver, CO) respectively.

Antioxidant Extraction

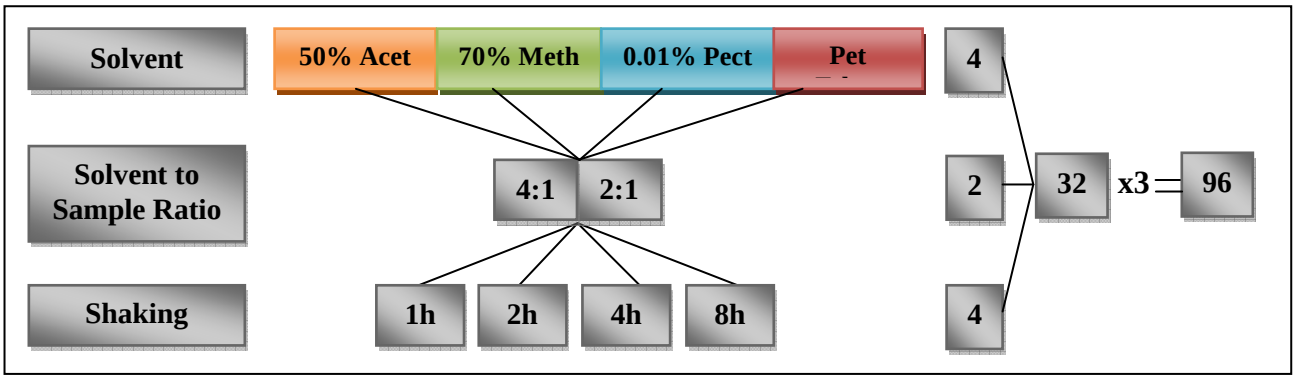
The solvents used to extract the antioxidants were:

- Petroleum Ether,
- 70% Methanol / Water,
- Water plus 0.01% pectinase and
- 50:50 Water / Acetone.

Each solvent was used on a 4:1 and 2:1 solvent to sample ratio and shaken at four (4) different time intervals of 1, 2, 4 and 8 hours; each combination of solvent;pomace ratio and extraction time was replicated three times.

Therefore, for each solvent eight (8) samples were utilized (2 ratios x 4 shaking times = 8 samples), with four (4) different solvents for a total of 32 samples per replication. Having three (3) replications we collected 3 x 32 = 96 samples (Figure 1).

Figure 1. Solvents, ratio and time used for antioxidant extraction of Cynthiana pomace.



Frozen ground tissue was thawed at room temperature for two hours and 20g samples were weighed into 125ml brown bottles (125mL Glass Amber with Teflon face lined cap, Fisherbrand, ThermoFisher Scientific Inc.) using an analytical balance (A-160, Denver Instruments Co). 20g were used to both facilitate adequate rinsing in later steps of the procedure and weighing limits of the balance.

Premixed solvents were added at the ratios mentioned in table 1 and bottles were caped and covered with parafilm. Samples were shaken using two identical water bath shakers (Classic C76, New Brunswick Scientific, Edison, NJ) for 1, 2, 4 and 8h at 250rpm.

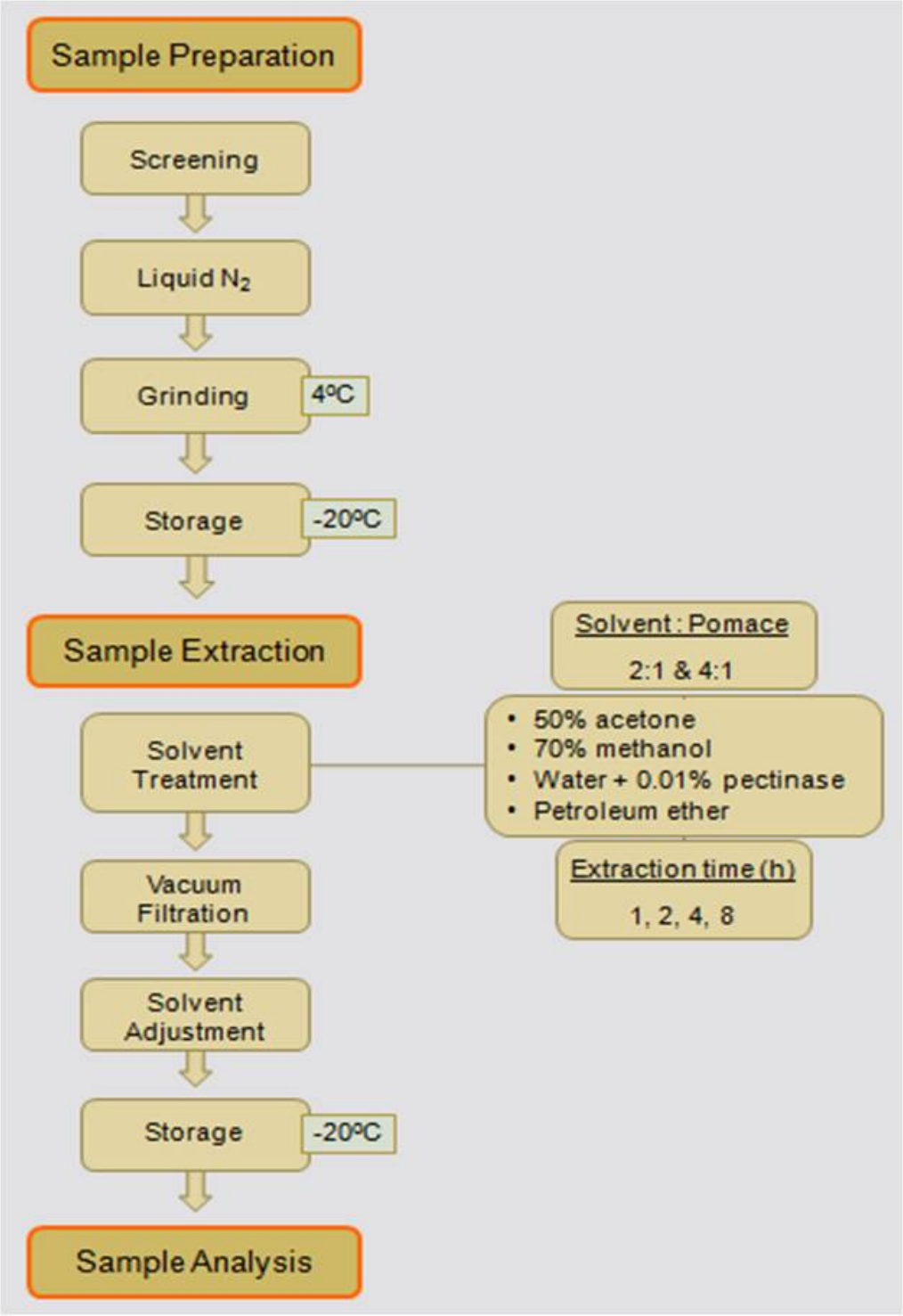
After shaking, samples were filtered under vacuum in a laminar flow hood, using a Cole Parmer flow meter (part # EW-32461-50) to assure minimal flow.

For filtration, a 5.5cm diameter Buchner funnel was used on a 250mL side arm Erlenmeyer flask through a #1 Whatman filter paper (55mm No1, Whatman Inc. Ltd., Mainstone, England). Samples were initially filtered until no visible dripping and then rinsed twice with approximately 10mL of solvent for two subsequent filtrations. The

filtrates were finally transferred to 100mL volumetric flasks and brought up to volume with the corresponding solvent, except the petroleum ether samples that were allowed to evaporate and re-suspended in 100% acetone. Samples were sealed with parafilm and stored at -20^{o}C until analysis.

An approximate summary of the procedure steps used for extraction of antioxidants from Cynthiana pomace is depicted below at Figure 2.

Figure 2. Extraction steps of Cynthiana (*Vitis aestivalis*) pomace.



ORAC Assay

All ORAC values were obtained on a Biotek Synergy2 microplate reader controlled by Gen5 software (version 1.04.5) (Bio-Tek Instruments, Inc., Winooski, VT). Sample plating and dilution was accomplished by a Precision 2000 automatic pipetting system managed by precision power software (version 1.0) (Bio-Tek Instruments, Inc.). Readings were carried out on BD Falcon 96well clear polystyrene microplates (Figure 3) (VWR International Inc., Bridgeport, NJ).

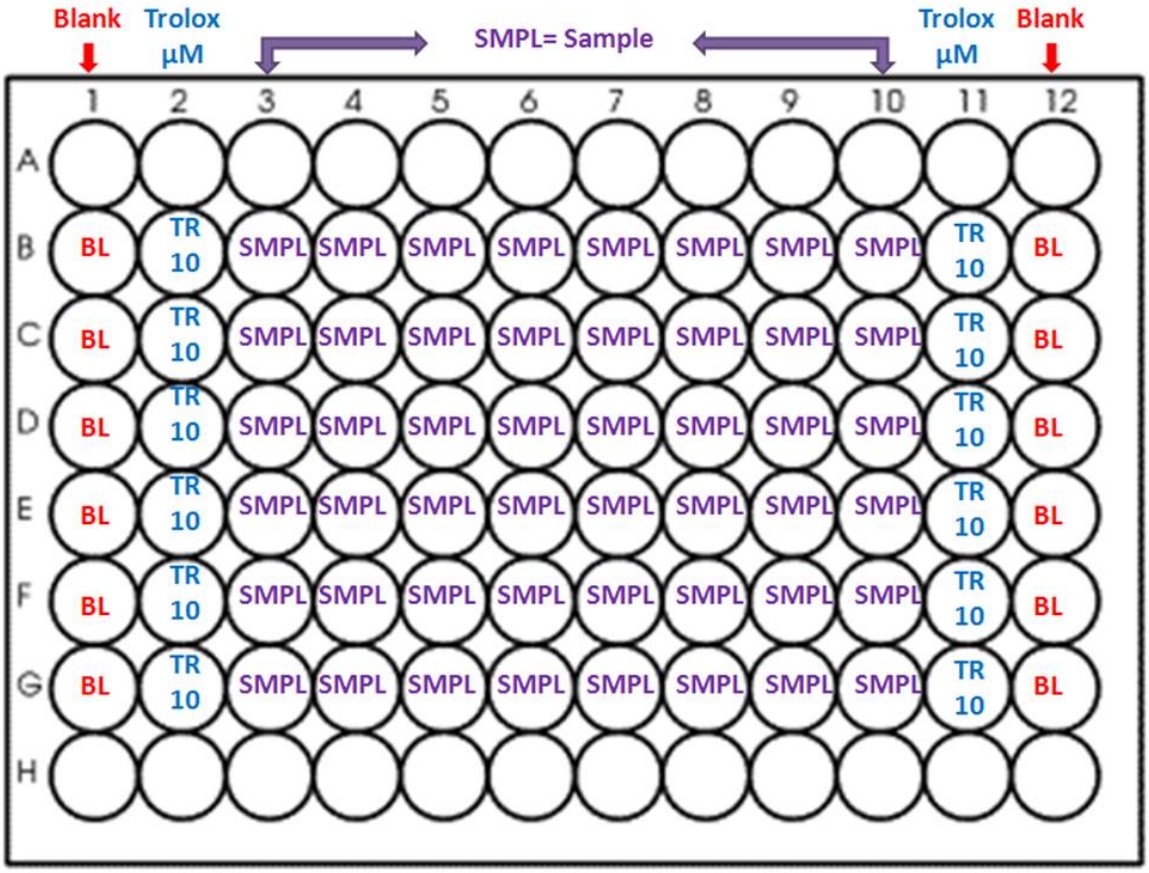
A modified procedure of Huang et al. (2002a, 2002b) and Ou et al. (2001) was used. Briefly, all reagents were prepared in 75mM phosphate buffer (pH 7.0). Fluorescein (FL) was used as a fluorescent probe and a target of free radical attack, with AAPH [2,2'-azobis(2-amidino-propane) dihydrochloride] being a peroxyl radical generator. The phosphate buffer was used as a blank and Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) at 10μM concentration was used as a standard. The phosphate buffer was also used for appropriate dilution of our more aqueous extracts (0.01% pectinase, 70% methanol, 50% acetone) before analysis. For the petroleum ether extracts (which were re-suspended in acetone), Trolox standards and additional sample dilutions were made with a mix of 7% RMCD (Randomly Methylated β-Cyclodextrin) in 50% acetone (Huang et al., 2002a). Samples were incubated for 10min at 37^{o}C and the Biotek reader was programmed to record fluorescence every two minutes after the addition of AAPH for 35 cycles (adequate time to allow >90 degradation of fluorescein). Fluorescence filters with an excitation wavelength of 485nm and an emission wavelength of 520nm were used. Results were obtained by calculating the Area Under the fluorescence decay Curve (AUC) for each of the Blank, Trolox, and Sample (Equation 1).

$$AUC = f_1/f_0 + \ldots f_i/f_0 + \ldots + f_{34}/f_0 + f_{35}/f_0 \quad (1)$$

where f_0 = initial fluorescence reading at 0 min and fi = fluorescence reading at time i.

By subtraction of the Blank area we compared the net areas of Trolox and Sample and by taking into account any dilution factor and sample weight, we express the final results as μmoles Trolox equivalent (TE) per gram of fresh pomace.

Figure 3. Reagent and sample layout on a 96 well plate for antioxidant activity of Cynthiana pomace.



HPLC analysis

Chemicals

High Performance Liquid Chromatography was used to identify and quantify the most prevalent antioxidants in Cynthiana grape pomace. The best combination from each solvent treatment/extraction was selected for this analysis using a preselected set of standards. HPLC grade Acetonitrile and o-phosphoric acid, were purchased from Fisher Scientific (Fair Lawn, NJ). Gallic acid, catechin monohydrate, caffeic acid, epicatechin, delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside, , coumaric acid, ferrulic acid, myricetin, resveratrol, quercetin hydrate, kaempherol and isorhamnetin were used at various concentrations to generate standard curves for sample analysis.

Chromatographic conditions

Polyphenol analysis was carried out by a modified procedure of Thimothe et al. (2007). We used a Dionex HPLC system (Dionex, Sunnyvale, CA) comprising of a Dionex P680 HPLC pump, a Dionex ASI-100 Automated Sample Injector, a Dionex TCC-100 Thermostatted Column Compartment and a Dionex Ultimate 3000 Photodiode Array detector. The system was controlled by Chromeleon software, version 6.80 Build 2212. The separation was performed with a Biorad RP-318 HiPore reversed phase C18 column (4.6mm x 250mm x 5µm) operated at 25°C, protected by a Dionex Acclaim 120 C18 guard cartridge (4.3mm x 10mm x 5µm i.d.). The mobile phases used were: solvent A) 0.1% phosphoric acid (H_3PO_4) in Milli-Q filtered water (RG Ultra-pure water system, Millipore Corp.), and solvent B) 0.1% H_3PO_4 in HPLC-grade acetonitrile. Data

acquisition was applied for 45min with a total run of 65min. Gradient elution was as follows: 0% B isocratic for 10min, from 0% B to 50% B in 40min, from 50% to 95% B in 1min, isocratic 95% B for 4min, gradient from 95% B to 0%B in 1min and isocratic at 0%B for 8min. Flow rate was 1mL/min, except at 95%B, which was at 1.5mL/min. The detector was set at 280, 320, 370, and 520 nm. Phenolic compounds were identified by comparison of retention times with the available standards.

Sample preparation

Polyphenol extraction was performed using a modified procedure of Thimothe et al. (2007). Pomace extracts (15mL) were evaporated under N_2 flow in $35^{O}C$ water bath (Zymark TurboVap, Zymark Center, Hopkinton, MA) to remove the organic solvent. The aqueous extract left, was passed through C18 Sep-Pak cartridges (WAT051910, Waters Corp., Milford, MA), which were preconditioned with 3mL methanol and followed by 10mL Milli-Q water. Compounds retained in the Sep-Pak were rinsed subsequently with 5mL Milli-Q water and 3mL 0.01N HCl. Cartridges were dried under N_2 flow for 2min and polyphenolic compounds were eluted with 3ml absolute methanol. Collected fraction were concentrated under N_2 flow in $35^{O}C$ water bath and resuspended in absolute methanol. Samples were filtered through 0.45μm nylon filters (Fisherbrand, PTFE, Fisher Scientific, Denver, CO) and injected for HPLC analysis.

RESULTS AND DISCUSSION

Cynthiana pomace composition

Pomace was analyzed upon arrival for moisture content, pH and water activity as described in materials and methods. Water activity of original Cynthiana pomace was 0.991 and average pH about 3.54; Main and Morris (2008) also reported pH range of 3.56-3.91 of Cynthiana grape juice as affected by different pruning methods. Results for % moisture content of original and treated pomace are shown below in Table 1.

Table 1. % Moisture content of original and freeze dried Cynthiana grape pomace.

			Drying Treatment				
	Sample ID	**Original**	**FD* 24h**	**FD 48h**	**FD 72h**	**FD** 96h**	**% Additional Moisture Loss from FD 24h**
Pomace	**Raw**	50.5	10.36	6.78	2.04	0.81	92
	Ground		15.18	6.23	3.65	2.44	84
	Skins		5.42	4.21	3.03	2.67	51
	Seeds		22.31	9.10	3.03	1.40	94

*** FD**= Freeze Dried, 80^{O}C, vacuum 15in. Hg
** Highlighted area applies to 96h FD

Moisture content of pressed residues from winemaking is dependent on several factors. Cultivar, vinification techniques, climate, and maturity stage are a few factors that contribute to moisture variability before processing. One of the major factors affecting the final moisture content of grape pomace is the degree of pressing during the winemaking process. Hang and Woodams (2008) reported 62-66% moisture content of

pomace from five New York sweet grape varieties, while Rubilar et al. (2007), a 55% and 60% from two different varieties. It was suggested that grape pomace intended to be used for distilled spirits (grappa, raki, tsipouro, tsikoudia, orujo etc.), should contain 55-70% moisture which allows extraction of organoleptic characteristics to the end product (Ruberto et al. 2008). Cynthiana pomace was found to contain about 50% moisture, which suggests that the variety was mainly used for wine production and was slightly overpressed.

Freeze drying (FD) of raw pomace was performed to investigate applicability for extraction methods as well as moisture levels. Skins and seeds were also tested for informational purposes only, since separation of these fractions was performed manually and further investigation would be out of the scope of this project. We found that FD of raw pomace reduced moisture content by 80% within 24h and up to 98 % in 96h. Seeds and ground pomace were still quite moist after 24h of FD with 22 and 15% moisture respectively, while skins, as expected, had the lowest (6%). Further FD was more beneficial for raw pomace and seeds, which showed an additional >90% reduction of moisture content in 96h (Table 1). This similarity is probably due to the presence of seeds in the raw pomace as well. Several research groups have also used FD as a processing step, with Altan et al. (2008) reporting a similar moisture content of ground pomace after 72h (3.9-6.3%).

Antioxidant activity

Antioxidant activity averaged over all extraction times and solvent:waste ratios was significantly different ($p<0.05$) between solvents, with 50% acetone (AC) demonstrating higher on average yield of 32μmoles TE/g tissue. The 0.01% pectinase (PC) and petroleum ether (PE) solvents were significantly less effective for extracting antioxidant compounds (Table 2). Methanol (MT) at 70% in water was also a good extracting medium for antioxidants, with average activity of 21μmoles TE/g pomace. Hogan et al. (2009) also used native Norton (Cynthiana) grapes from Virginia to determine their antioxidant profile, and reported an average of 23μM TE/g tissue for methanolic extracts of deseeded grapes, which compares to our findings (Table 2).

Table 2. Average antioxidant activity of Cynthiana pomace extracts.

Solvent	Average Activity (μmoles TE/gr tissue)
50% Acetone	32[a]
70% Methanol	21[b]
0.01% Pectinase	5[c]
Petroleum Ether	2[d]

Activity shown is an average across all shaking levels and ratios of solvent

[a] Numbers with different letters denote significance at p<0.05

The best ratio of solvent to sample for the highest yield of antioxidants was 4:1 for all solvents except for petroleum ether where ratio wasn't significant (Table 3). The expected antioxidant activity in pomace is in majority due to polyphenolic compounds, which are in general more polar; our results indicate that the more lipophillic antioxidants exist in small amounts in Cynthiana pomace (shown as low antioxidant activity) and

therefore efficiently extracted by the non polar nature of PE regardless the ratios used in this experiment.

Table 3. Influence of solvent ratio on antioxidant activity of Cynthiana pomace extracts.

Solvent	Solvent : Sample Ratio		
	4 : 1	2 : 1	
50% Acetone	34.8[a]	29.8[b]	Average Antioxidant Activity (µmoles TE/gr tissue)
70% Methanol	22.2[c]	19.8[d]	
0.01% Pectinase	5.2[e]	4.8[f]	
Petroleum Ether	1.9[g]	1.9[g]	

[a] Numbers with different letters denote significance at p<0.05.

The highest yield of antioxidants was slightly above the average for AC and MT extracts, while for PC and PE no difference was observed (Table 4 vs Table 2).

Table 4. Highest antioxidant activity for the best treatment of each solvent.

Solvent	Ratio	Shaking time (h)	Highest Activity (µmoles TE/gr tissue)
50% Acetone	4 : 1	2	36.9[a]
70% Methanol	4 : 1	2	23.0[b]
0.01% Pectinase	4 : 1	2	5.2[c]
Petroleum Ether	2: 1	2	2.0[d]

[a] Numbers with different letters denote significance at p<0.05

Increasing the duration of the extraction time did not follow a linear type of regression for antioxidant activity. For AC extracts there was no difference between 2 and 8 hours of shaking for maximum antioxidant extraction, while for all the other solvents no difference was observed for extractions above 2 hours of shaking (Table 5).

The lowest extraction time (1h) was also among the lowest antioxidant activities for all solvents. Therefore we may conclude that the optimum duration time for antioxidant extraction from Cynthiana pomace is 2h of shaking.

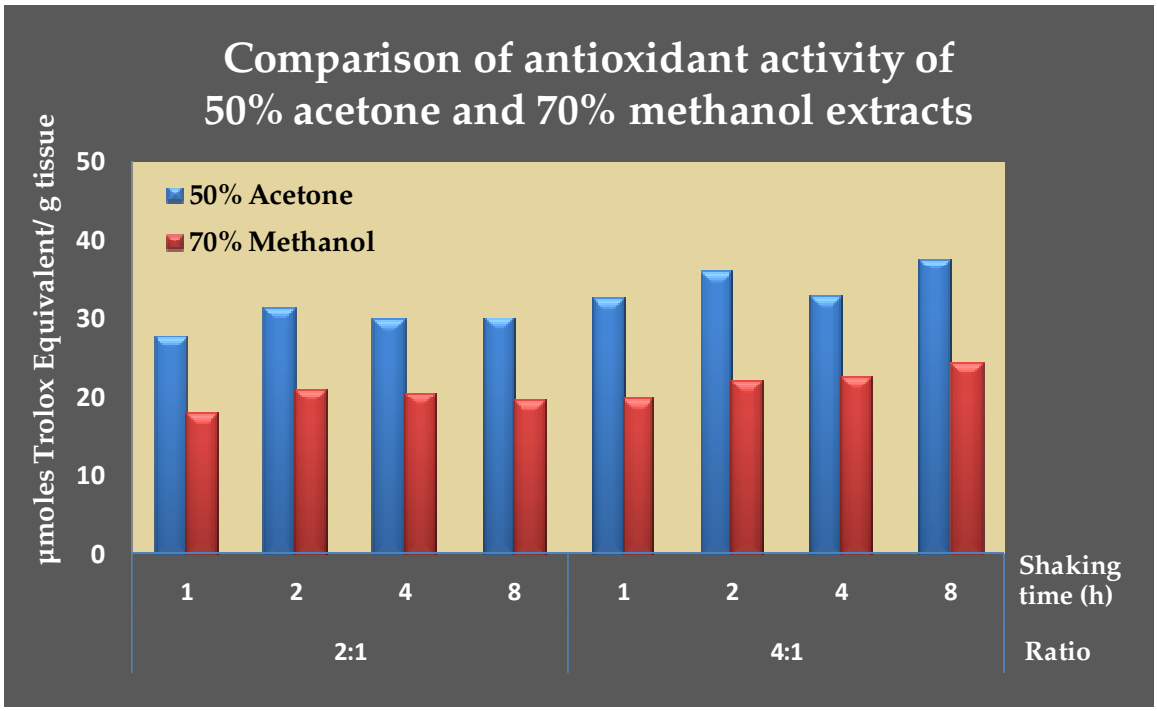
Table 5. Influence of shaking time on antioxidant activity of Cynthiana pomace extracts.

Solvent	Shaking time (h)	Average Activity (μmoles TE/gr tissue)
50% Acetone	8	33.9[a]
	2	33.8[a]
	4	31.4[b]
	1	30.1[b]
70% Methanol	8	22.1[c]
	4	21.6[c]
	2	21.5[c]
	1	18.9[d]
0.01% Pectinase	2	5.2[e]
	4	5.1[e]
	8	5.0[ef]
	1	4.7[f]
Petroleum Ether	2	2.0[g]
	8	1.9[gh]
	4	1.8[gh]
	1	1.8[h]

[a] **Numbers with different letters denote significance at p<0.05**

Between our two best solvents used (AC and MT) for pomace antioxidant screening, AC proved to be a more efficient extracting medium among all treatments when compared to MT, increasing antioxidant extraction by 45-60% (Figure 4).

Figure 4. Antioxidant yields from Cynthiana pomace of 50% Acetone and 70% Methanol.



In addition AC has shown very good yields for antioxidants even when used at half the ratio of solvent to sample (Table 6), indicating that it can be a very good solution for antioxidant extraction from grape pomace with less solvent used. This might be of greater importance in a larger scale industrial extraction where cost and efficiency are more detrimental factors.

Table 6. Influence of ratio on the highest ORAC values for 50% Acetone.

Solvent	Treatment	Average µmoles TE/gr tissue
50% Acetone	4 : 1 ratio, 2h shaking	36.1[a]
	2 : 1 ratio, 2h shaking	31.6[b]

[a] Numbers with different letters denote significance at $p<0.05$.

Our results indicate that Cynthiana pomace has notable antioxidant activity and could be a potential source of natural compounds for the pharmaceutical and food industry. When compared to common known antioxidant sources, Cynthiana pomace extracts rank well among them (Table 7).

Table 7. ORAC antioxidant activity from different food sources.

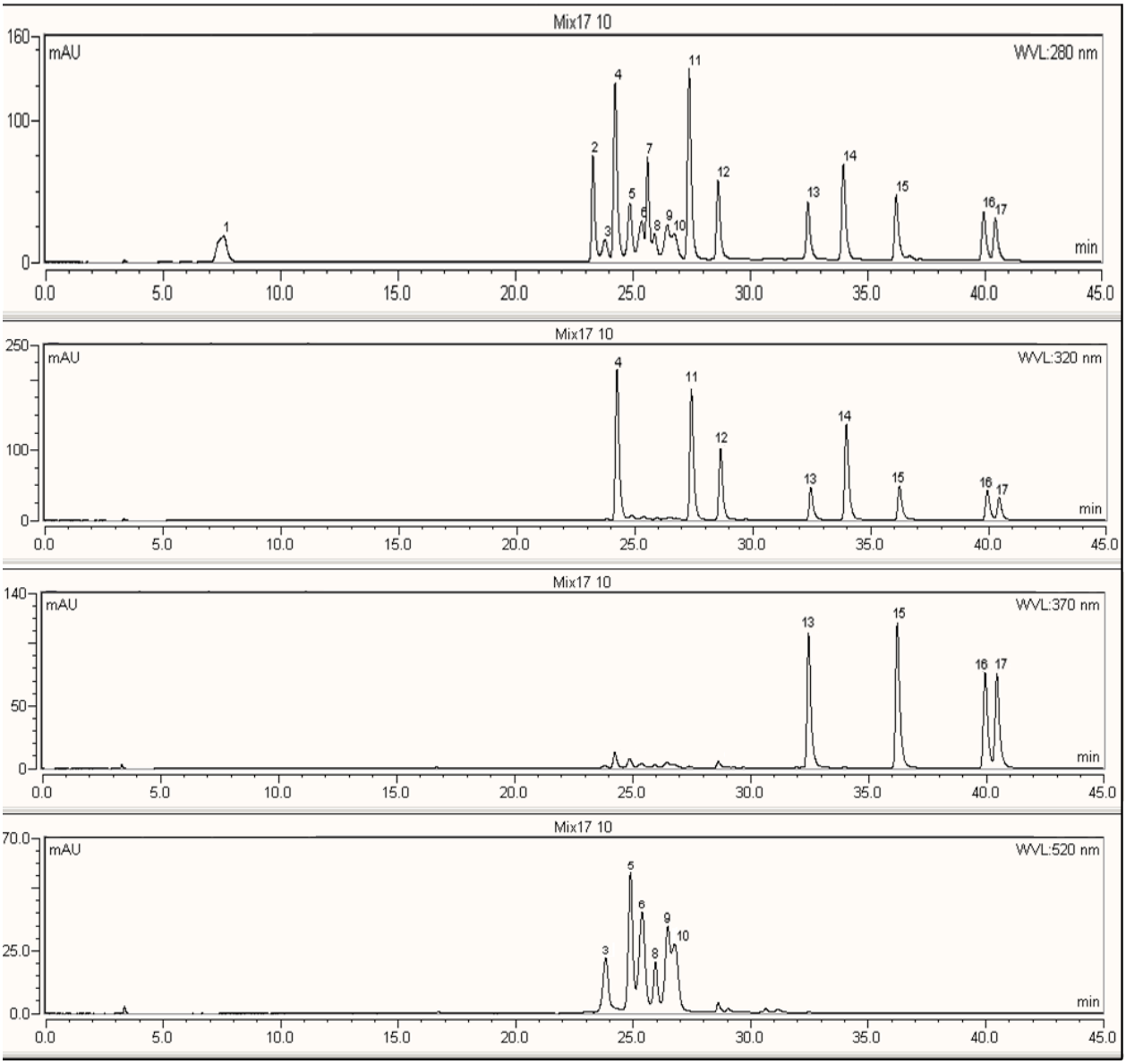
Food Source/products	Total ORAC (µmoles TE/ **100 gr** tissue)
Blackberries, raw	5347
Blueberries, raw	6552
Grapes, red, raw	1260
Grapes, white or green, raw	1118
Grape juice, white	793
Grape juice, red	1788
Tomatoes, raw and cooked	367-694
Cynthiana pomace (our trial)	≈2000-3700

Values obtained from USDA 'ORAC of selected foods - 2007'

HPLC – Compound identification

The best method employed for antioxidant extraction (50% acetone, 2h, and 4:1 ratio) was analyzed in HPLC for major compound identification using the available standards mentioned above in the materials and methods section. Representative chromatograms of all the standards used are shown below in Figure 5, with individual chromatograms of standards used for separation of phenolic acids (280nm, 320nm), anthoxanthins, stilbenes (280nm, 370nm) and anthocyanins (520nm). Retention times are shown in Table 8.

Figure 5. HPLC chromatograms of standards mixture at 280, 320, 370 and 520nm.



Peak assignment: 1. Gallic acid, 2. Catechin, 3. Delphinidin-3-O-glucoside, 4. Caffeic acid, 5. Cyanidin-3-O-glucoside, 6. Petunidin-3-O-glucoside, 7. Epicatechin, 8. Pelargonidin-3-O-glucoside, 9. Peonidin-3-O-glucoside, 10. Malvidin-3-O-glucoside, 11. Coumaric acid, 12. Ferrulic acid, 13. Myricetin, 14. Resveratrol, 15. Quercetin, 16. Kaempherol and 17. Isorhamnetin.

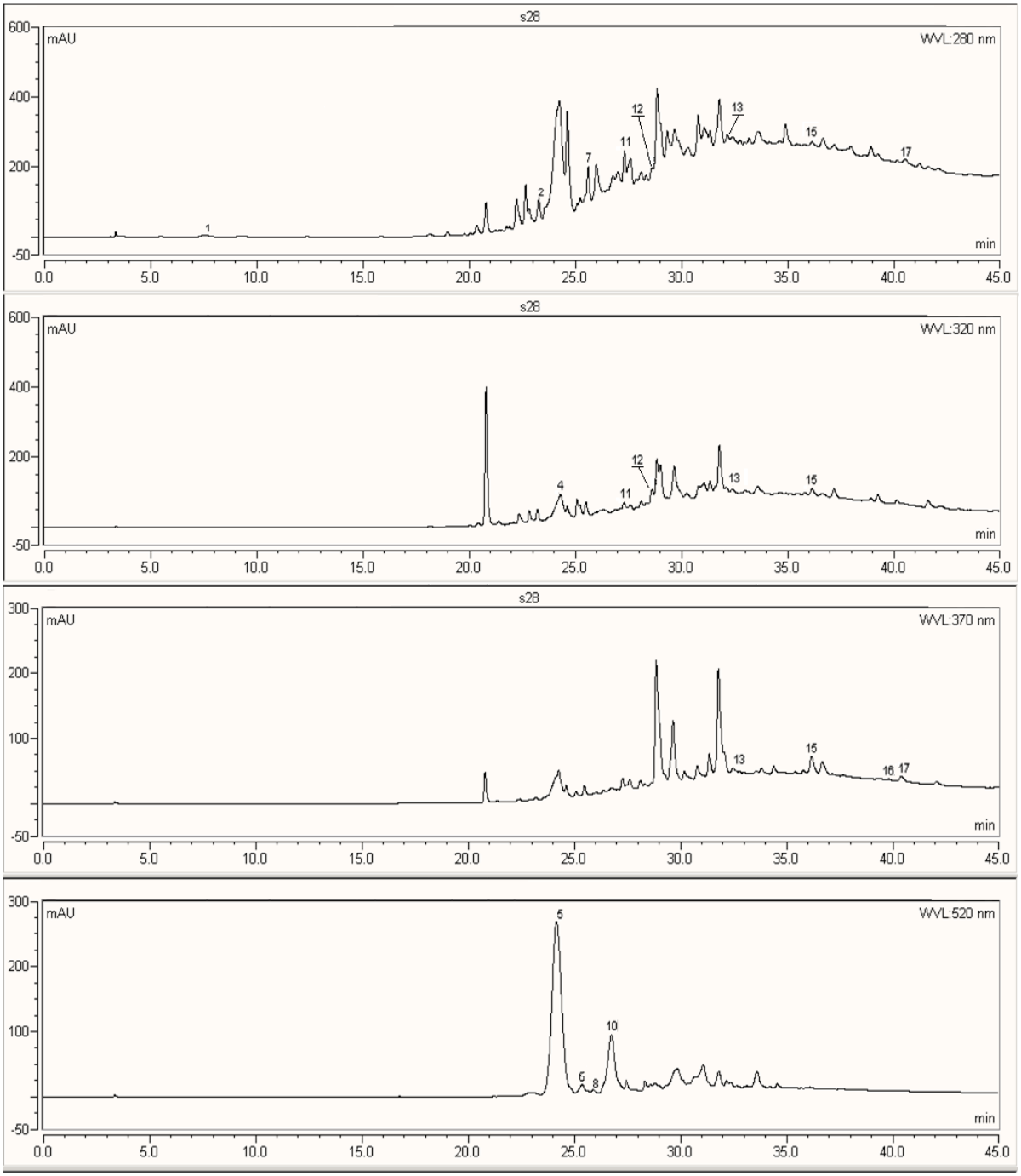
Table 8. Retention time of HPLC standards.

No.	Peakname	Ret.Time min
1	Gallic Acid	7.583
2	Catechin	23.317
3	Delphinidin 3-O-Glu	23.833
4	Caffeic	24.258
5	Cyanidin 3-O-Gluc	24.883
6	Petunidin 3-O-Gluc	25.383
7	Epicatechin	25.642
8	Pelargonidin 3-O-Gl	25.942
9	Peonidin 3-O_Gluc	26.475
10	Malvidin 3-O-Gluc	26.767
11	Coumaric	27.408
12	Ferrulic	28.642
13	Myricetin	32.467
14	Resveratrol	33.975
15	Quercetin	36.225
16	Kaempherol	39.950
17	Isorhamnetin	40.450

Analysis of Cynthiana pomace extracts showed that the major compounds identified were cyanidin 3-*O*-glucoside and malvidin 3-*O*-glucoside; pomace consisting of skins and seeds, is consequently rich in anthocyanins, as other researchers have also noted (Rababah et al. 2008). Cho et al (2004) have also reported a major peak for cyanidin 3-*O*-glucoside in Cynthiana, as opposed to other genotypes were malvidin 3-*O*-glucoside is greater. Epicatechin and coumaric acid were present in lesser amounts, and caffeic acid, quercetin and catechin were also detected, along with traces of other compounds (Figure 6).

Several major peaks weren't identified at 320 and 370nm with our set of standards, suggesting that other compounds are present in significant amounts in Cynthiana pomace and may be worth further investigation. In addition, we must consider the possibility of other peaks (compounds) overlapping at the same retention times, which we could not detect using our method without the use of an in-line mass spectrometer.

Figure 6. Typical HPLC chromatograms of 50% Acetone extracts from Cynthiana pomace.



Peaks assignment: 1. Gallic acid, 2. Catechin, 4. Caffeic acid, 5. Cyanidin-3-O-glucoside, 6. Petunidin-3-O-glucoside, 7. Epicatechin, 8. Pelargonidin-3-O-glucoside, 10. Malvidin-3-O-glucoside, 11. Coumaric acid, 12. Ferrulic acid, 13. Myricetin, 15. Quercetin, 16. Kaempherol and 17. Isorhamnetin.

CONCLUSIONS

Antioxidant compounds increasingly have been of interest in the last decade. Public awareness of these compounds has also increased along with an increased demand for food additives from natural sources. Fruit and vegetable processing industries generate large amounts of waste, which may contain valuable byproducts of interest. A great deal of research has been done on grape and wine waste streams, which has demonstrated the presence of significant amounts of antioxidants.

Our method presents a potentially industrial scale, single-solvent extraction process for screening antioxidants from grape pomace – and possibly other waste streams. Cynthiana pomace was used as a model for our purposes and 50% Acetone/Water solvent proved to be a good extraction medium when used for 2h at 2:1 solvent:sample ratio. Yield of 31μmoles TE/g tissue suggest that Cynthiana pomace is a good source of natural antioxidants, with anthocyanins cyanidin 3-*O*-glucoside and malvidin 3-*O*-glucoside being two of the major compounds in the extracts.

REFERENCES

Altan, A., M. Maskan and K.L. McCarthy. 2008. Twin-screw extrusion of barley-grape pomace blends: Extrudate characteristics and determination of optimum processing conditions. Journal of food engineering. 89 (1):24-32.

Brenes, A., S.G. S Lyago-Ayerdy, I. Arija, F. Saura-Calixto, A. Viveros, I. Go li and C. Centeno. 2008. Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens. Poultry science. 87(2):307-316.

Cho, M.J., J.R. Clark, R.L. Prior and L.R. Howard. 2004. Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. Journal of the Science of Food and Agriculture. 84 1771-1782.

Food and Agriculture Organization of the United Nations -- Food and Agricultural commodities production. 2009. http://faostat.fao.org/site/567/default.aspx#ancor (17 November, 2009).

Hang, Y.D. and E.E. Woodams. 2008. Methanol content of grappa made from New York grape pomace. Bioresource technology. 99 (9):3923-3925.

Hogan, S., B. Zoecklein, K. Zhou, L. Zhang and J. Li. 2009. Antioxidant properties and bioactive components of Norton (Vitis aestivalis) and Cabernet Franc (Vitis vinifera) wine grapes. Food science and technology. 42 1269-1274.

Huang, D., B. Ou, M. Hampsch-Woodill, J.A. Flanagan and E.K. Deemer. 2002a. Development and validation of oxygen radical absorbance capacity assay for

lipophilic antioxidants using randomly methylated beta-cyclodextrin as the solubility enhancer. Journal of agricultural and food chemistry. 50 1815-1821.

Huang, D., B. Ou, M. Hampsch-Woodill, J.A. Flanagan and R.L. Prior. 2002b. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. Journal of agricultural and food chemistry. 50 4437-4444.

Hung, L.M., J.K. Chen, S.S. Huang, R.S. Lee and M.J. Su. 2000. Cardioprotective effect of resveratrol, a natural antioxidant derived from grapes. Cardiovasc Res. 47(3):549-55.

Kallithraka, S., A.A.A. Mohdaly, D.P. Makris and P. Kefalas. 2005. Determination of major anthocyanin pigments in Hellenic native grape varieties (Vitis vinifera sp.): association with antiradical activity. Journal of food composition and analysis an official publication of the United Nations University, International Network of Food Data Systems. 18 (5):375-386.

Leblanc, M.R., P.W. Wilson and C.E. Johnson. 2008. Influence of pressing method on juice stilbene content in Muscadine and Bunch grapes. Journal of food science an official publication of the Institute of Food Technologists. 73(4):h58-h62.

Llobera, A. and J. Canellas. 2007. Dietary fibre content and antioxidant activity of Manto Negro red grape (vitis vinifera): Pomace and stem. Food chemistry. 101:659-666.

Main, G.L. and J.R. Morris. 2008. Impact of Pruning Methods on Yield Components and Juice and Wine Composition of Cynthiana Grapes. American journal of enology and viticulture. 59 (2):179-187.

Ou, B., R.L. Prior and M. Hampsch-Woodill. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. Journal of agricultural and food chemistry. 49 4169-4626.

Rababah, T.M., K.I. Ereifej, M.A. Al-Mahasneh, K. Ismaeal, A.-G. Hidar and W. Yang. 2008. Total phenolics, antioxidant activities, ans anthocyanins of different grape seed cultivars grown in Jordan. International Journal of Food Properties. 11 472–479.

Ruberto, G., A. Renda, V. Amico and C. Tringali. 2008. Volatile components of grape pomaces from different cultivars of Sicilian Vitis vinifera L. Bioresource technology. 99 (2):260-268.

Rubilar, M., M. Pinelo, C. Shene, J. Sineiro and M.J. Nunez. 2007. Separation and HPLC-MS Identification of Phenolic Antioxidants from Agricultural Residues: Almond Hulls and Grape Pomace. Journal of agricultural and food chemistry. 55 (25):10101-10109.

Su, H.C., L.M. Hung and J.K. Chen. 2006. Resveratrol, a red wine antioxidant, possesses an insulin-like effect in streptozotocin-induced diabetic rats. Am J Physiol Endocrinol Metab. 290(6):E1339-46.

Thimothe, J., I.A. Bonsi, O.I. Padilla-Zakour and H. Koo. 2007. Chemical Characterization of Red Wine Grape (*Vitis vinifera* and *Vitis* Interspecific Hybrids) and Pomace Phenolic Extracts and Their Biological Activity against *Streptococcus mutans*. Journal of agricultural and food chemistry. 55 (25):10200-10207.

Valiente, C., E. Arrigoni, R.M. Esteban and R. Amado. 1995. Grape pomace as a potential food fiber. Journal of Food Science. 60:818-820.

Zhao, J., J. Wang, Y. Chen and R. Agarwal. 1999. Anti-tumor-promoting activity of a polyphenolic fraction isolated from grape seeds in the mouse skin two-stage initiation-promotion protocol and identification of procyanidin b5-3'-gallate as the most effective antioxidant constituent. Carcinogenesis. 20(9):1737-45.

CHAPTER IV

Experiment II

Antioxidant profile and analysis of Cabernet franc grapes, pomace, and wine during various steps of wine production.

ABSTRACT

Wine popularity and production have increased in the US during the last decade, and with more states involved in the process, greater volumes of waste are being generated. Valuable compounds have been identified in the waste products of winemaking (pomace) and efforts at identification and concentration of these compounds have been made by several research groups. Our study focused on recovery and identification of the major antioxidant compounds in Cabernet franc grapes during small-scale wine production. Measures of antioxidant activity using Oxygen Radical Absorbance Assay (ORAC) were performed and correlated with total phenolic content (TPC). High Performance Liquid Chromatography (HPLC) analysis was also conducted to identify major phenolics present. Ninety kg of Cabernet franc grapes were slightly crushed during a desteming process and placed in two industrial stainless steel vessels for fermentation with the addition of wine yeast (*saccharomyces cerevisiae*) and yeast nutrients. After fermentation to dryness, wine and pomace were separated, with wine

transferred to 19L glass carboys and pomace pressed with a small, industrial-scale hydraulic press. Pressed pomace was frozen at -20^{O}C until analysis and collected juice was added to the corresponding glass carboy. Pomace tissue was extracted with four different solvent combinations (previously developed) of 50% acetone (AC) and 70% methanol (MT) and analyzed with ORAC, TPC and HPLC. Wine samples were collected on week1 (immediately after pressing) and on week15 after fermentation and during stabilization, and subjected to similar analysis.

Antioxidant activity of Cabernet franc pomace was higher on AC than MT extracts, with yields of approximately 83 and 56μmoles Trolox Equivalents/g pomace (TE/g). Antioxidant activity was positively correlated with TPC (r=0.96) with average content of 2.6mg and 1.2mg Gallic Acid Equivalents/g tissue (GAE/g) for AC and MT extracts respectively.

Wine had also good antioxidant properties with average 27μmoles TE/mL and showed slightly higher values on week15 than week1. TPC was also correlated with antioxidant activity (r=0.86) with average content of 762mg GAE/L of wine. The major compounds in wine identified with HPLC were predominately gallic acid and epicatechin, with peonidin and malvidin 3-*O*-glucosides in smaller amounts. Caffeic, coumaric and ferrulic acids were also identified, along with traces of myricetin, quercetin, kaempherol and isorhamnetin.

Pomace extracts contained mainly catechin and epicatechin, with ferrulic acid, quercetin and isorhamnetin. A major peak identified for anthocyanins was probably a combination of peonidin 3-*O*-glucoside and malvidin 3-*O*-glucoside. Both cabernet franc

pomace and wine proved to have good antioxidant activities, with pomace being a more potent valuable source of natural biomolecules.

MATERIALS AND METHODS

Chemicals

ORAC: Potasium phosphate dibasic (K_2HPO_4) ACS (EM Science, US)), sodium phosphate monobasic ($NaHPO_4*H_2O$) ACS (Fisher Scientific NJ, US), AAPH [2,2'-azobis(2-amidino-propane) dihydrochloride] (Waco Chemicals Inc., Richmond, VA), Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Fluka Chemika, Switcherland), fluorescein disodium (Sigma-Aldrich, St-Louis, MO), Randomly Methylated β-Cyclodextrin (RMCD)(Cyclodextrin Technologies Development Inc., www.cyclodex.com).

HPLC: HPLC grade solvents and analytical reagent grade ortho-phosphoric acid were purchased from Fisher Scientific. Deionized water was produced by a Milli-Q unit (Millipore, Bedford, MA, USA). Gallic acid, catechin monohydrate, epicatechin, caffeic acid, coumaric acid, ferrulic acid, myricetin, quercetin, trans-resveratrol, kaempferol and isorhamnetin were purchased from Fisher Scientific (Fair lawn,NJ); cyanidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside were obtained from Polyphenols Laboratories AS (Sandnes, Norway).

Miscellaneous: Folin-Ciocalteu reagent (Fluka BioChemica, Switzerland), gallic acid and sodium carbonate (Sigma Aldrich, St. Louis, MO), Lalvin 71B-112 yeast (*saccharomyces cerevisiae*) (Lallemand Inc, Monteal, Canada), Fermaid wine yeast nutrient and potassium metabisulfite (Presque Isle Wine Cellars, North East PA), L-Tartaric acid (Fisher Scientific).

Samples

Cabernet franc whole grapes were obtained from the Oklahoma State University research vineyard located on the Cimarron Valley Research Station in Perkins, Oklahoma and transferred in laboratory facilities in Stillwater, OK. Upon arrival, samples were immediately placed at a -20^{o}C freezer until further processing

Winemaking and sampling

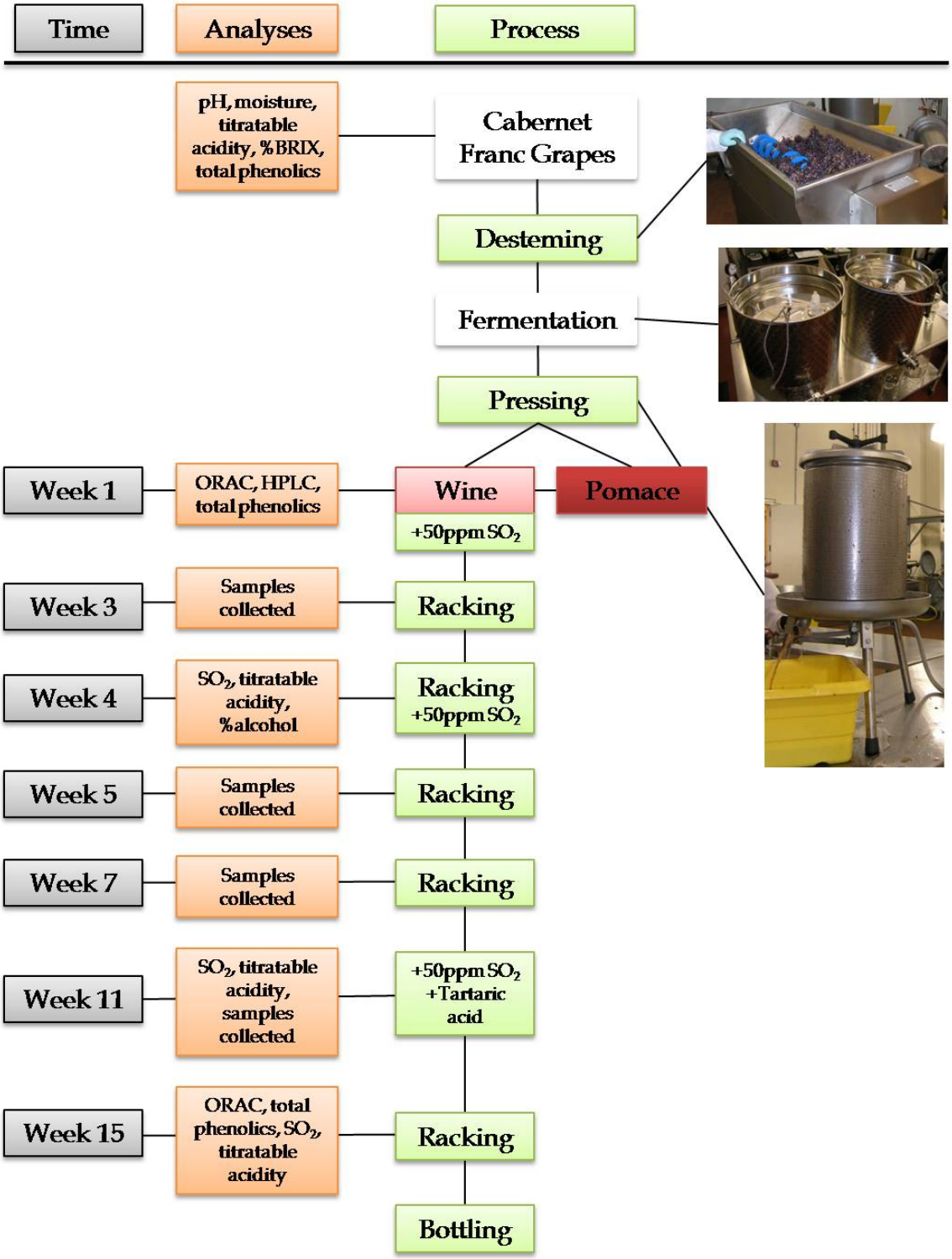
Grapes were thawed overnight at room temperature and equally divided in two replicates (≈45kg each). Samples were collected for initial analyses of pH, % soluble solids (BRIX), titratable acidity, total phenolics, and moisture content. The two replicates were lightly crushed for stem separation using small scale commercial destemmer-crusher (Jolly-60, St. Patrick's of Texas, Austin, TX) and placed into two 100 liter (25 gallon) stainless steel fermentation vessels. Wine grade yeast (*saccharomyces cerevisiae*) and yeast nutrient (Fermaid) was also added. Vessels were capped with adjustable height lids allowing approximately 25cm (≈10 inches) of headspace. Samples fermented to dryness, monitored by rapid residual sugar tests (AV-RS Accuvin LLC, Napa, CA). Cap was punched down daily until fermentation was completed.

After fermentation, samples were pressed using a small scale table top water-powered bladder press (Zampelli Enotech JRL, Italy), which allowed separation of juice/wine and pomace. Samples of wine and pomace were collected for alcohol content, total phenolics, ORAC, HPLC and were stored at -20^{o}C (Week1 sampling).

Wine from each replication was transferred to a 19liter (5 gallon) glass carboy allowing minimal headspace remainder volumes were combined in a separate glass

carboy. Potassium metabisulfite ($K_2S_2O_4$) at 50ppm was added to each carboy to prevent microbial growth and oxidation, followed by headspace flushing with nitrogen to remove oxygen. Wines were allowed to settle and were racked every two weeks until no further sediment was observed. At the same time intervals, samples of wine were collected from Week1 to Week15, while titratable acidity, pH and the free and bound SO_2 were monitored. An approximate summary of the wine processing is depicted below in Figure 7.

Figure 7. Cabernet franc wine processing flow and sampling.



Pomace handling

Pomace samples were initially collected after pressing for total phenolics and moisture analysis; remaining sample material was immediately frozen at -20^{o}C until further processing. Optimal extraction protocols as identified in Experiment I (50% Acetone, 4:1, 8h & 2h shaking, 70% Methanol 4:1, 8h & 4h shaking) were used to extract antioxidants from pomace. Extraction steps and sampling were done in triplicates as described in Experiment I.

Total phenolics content

For whole grapes and pomace an extraction step for total phenolics was performed prior to analysis. Briefly, grape and pomace tissue was homogenized using a Waring blender (model 51BL310) and extracted with a solvent mixture of 40/40/20/0.1% of Acetone/Methanol/Water/Acetic Acid respectively. 25mL of solvent were added to 5g of homogenized sample and incubated in an agitated water bath of 60^{o}C for 1h. Samples were allowed to cool in room temperature and further homogenized using a polytron tissuemizer (PowerGen 700, Fisher scientific) for 30 seconds. Samples were coarse filtered with Miracloth (make) and frozen at -20^{o}C until analysis. For wine samples, a direct volume of wine was used for total phenols determination as described below.

The total phenol content (TPC) was determined by a modified method of Singleton and Rossi (1965). Specifically, 0.5 mL of extract was mixed with 5mL of distilled water and 1 mL of Folin–Ciocalteu reagent into 25mL volumetric flask and let stand for 5-8 minutes. 10mL of 7% sodium carbonate were added, brought up to volume with DI water and let stand for 2h. Absorbance was measured at 765nm and TPC was

expressed as Gallic acid equivalent (GAE)/g of extract or GAE/L of wine. A calibration curve using Gallic acid in the extraction solvent was also created using concentrations of 0.125, 0.25, 0.5, and 1.0mg/mL.

Free and total SO_2

Free and bound SO_2 were estimated using an Oxidation/Aeration apparatus (make) as follows: 20mL of wine sample were placed into the round bottom flask and 10mL of 25% phosphoric acid were added along with 3-4 boiling beads and the bubbler/stopper. 10mL of 3% Hydrogen Peroxide were transferred to the impinger and three drops of SO_2 indicator (0.1% Methyl Red + 0.05% Methylene Blue, Presque Isle Wine Cellars, NorthEast PA) were added. If color was too purple or too green, it was adjusted to gray-green with dilute NaOH or HCL respectively. Free SO_2 was determined by the pH change in the impinger (color change to light purple), when the apparatus was operated for 10min under light vacuum (1Lt/min) and subsequent titration with 0.01N NaOH until the initial grey-green color was achieved. Calculations were:

Free SO_2 (ppm) = N NaOH x mls NaOH x 1600

Bound SO_2 was calculated for the same sample, after a new set of reagents was placed into the impinger and heat was applied to the sample for 15min under vacuum as above. Similarly, with titration of 0.01N NaOH to the initial grey-green color and using the same calculation as above, we estimated the bound SO_2. Total SO_2 was calculated by the addition of free and bound SO_2.

Wines were tested periodically for SO_2 levels and were adjusted accordingly to 50-60ppm of free SO_2 with potassium metabisulfite. All estimations were performed with

the above procedure except the last sampling of Week15 were free SO2 was determined with a test kit (Titrets, Chemetrics, Calverton, VA).

Titratable acidity, pH, soluble solids and alcohol content

Titratable acidity of grape juice and wines was performed on a Titrando 809 (Metrohm USA, Riverview, FL) automatic titrator, using 5mL of sample in 100mL deionized water and titrated with 0.1N NaOH to an endpoint of pH=8.2. Results were expressed as % tartaric acid, or mg/L tartaric acid.

Soluble solids and pH were obtained using a Leica Auto Abbe 10500 bench top refractometer (Reichert Analytical Instruments, Depew, NY) and an Accumet pH meter (AB15 Basic, Fisher Scientific, Denver, CO) respectively. Titratable acidity was also monitored periodically to ensure adequate levels for microbial growth prevention and SO_2 adjustment. During our experiment, acidity was adjusted once with food grade tartaric acid to an approximate pH=3.8.

Alcohol content was determined by the use of an ebulliometer (Electric Ebulliometer, Dujardin-Salleron Laboratories, Arcueil, France) after the completion of fermentation.

ORAC Assay

All ORAC values were obtained on a Biotek Synergy2 microplate reader controlled by Gen5 software (version 1.04.5) (Bio-Tek Instruments, Inc., Winooski, VT). Sample plating and dilution was accomplished by a Precision 2000 automatic pipetting

system managed by precision power software (version 1.0) (Bio-Tek Instruments, Inc.). Readings were carried out on a BD Falcon 96well clear polystyrene microplates (VWR International Inc., Bridgeport, NJ) (Figure 8).

A modified procedure of Huang et al. (2002a, 2002b) and Ou et al. (2001) was used. Briefly, all reagents were prepared in 75mM phosphate buffer (pH 7.0). Fluorescein (FL) was used as a fluorescent probe and a target of free radical attack, with AAPH [2,2'-azobis(2-amidino-propane) dihydrochloride] being a peroxyl radical generator. The phosphate buffer was used as a blank and Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) at concentrations of 10, 20, 30, 40, 50μM was used to create a standard curve. The phosphate buffer was also used for appropriate dilution of the extracts (50% acetone, 70% methanol, and wine) before analysis.

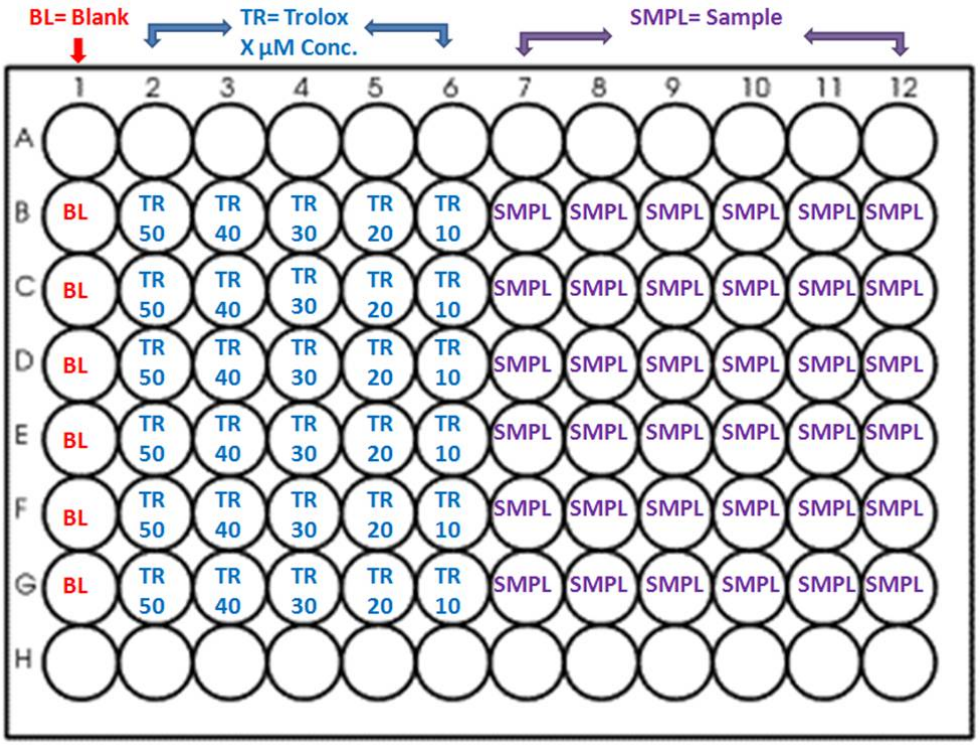
Samples (20μL), Trolox at the above concentrations (20μL), and blank (20μL of phosphate buffer) were added to the 96well plate according to the layout of figure 5. Fluorescein at 160μL (0.6μM) was added to all wells and the plate was incubated for 10min at 37^{o}C inside the Biotek reader. After the addition of AAPH (20μL of 200mM) to each well, fluorescence was recorded every two minutes for 35 cycles (adequate time to allow >90 degradation of fluorescein), at excitation wavelength of 485nm and an emission wavelength of 520nm. Results were obtained by calculating the Area Under the fluorescence decay Curve (AUC) for each of the Blank, Trolox, and Sample (Equation 1).

$$AUC = f_1/f_0 + \ldots f_i/f_0 + \ldots + f_{34}/f_0 + f_{35}/f_0 \quad (1)$$

where f_0 = initial fluorescence reading at 0 min and fi = fluorescence reading at time i.

By subtraction of the Blank area we compared the net areas of Trolox and Sample and by taking into account any dilution factor and sample weight, we express the final results as μmoles Trolox equivalent (TE) per gram of fresh pomace.

Figure 8. Reagent and sample layout on a 96 well plate for antioxidant activity of wines and pomace from Cabernet franc.



HPLC Analysis

Chemicals

High Performance Liquid Chromatography was used to identify and quantify the most prevalent antioxidants in Cabernet franc grape pomace and wine samples. Chemicals used for creating standard curves included: Gallic acid, catechin monohydrate, caffeic acid, epicatechin, cyanidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, peonidin-3-

O-glucoside, petunidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, coumaric acid, ferrulic acid, myricetin, resveratrol, isorhamnetin, kaempherol and quercetin hydrate were used at various concentrations to generate standard curves for sample analysis.

Chromatographic conditions

Polyphenol analysis was carried out by a modified procedure of Thimothe et al. (2007). We used a Dionex HPLC system (Dionex, Sunnyvale, CA) comprising of a Dionex P680 HPLC pump, a Dionex ASI-100 Automated Sample Injector, a Dionex TCC-100 Thermostatted Column Compartment and a Dionex Ultimate 3000 Photodiode Array detector. The system was controlled by Chromeleon software, version 6.80 Build 2212. The separation was performed with a Biorad RP-318 HiPore reversed phase C18 column (4.6mm x 250mm x 5μm) operated at 25°C, protected by a Dionex Acclaim 120 C18 guard cartridge (4.3mm x 10mm x 5μm i.d.). The mobile phases used were: solvent A) 0.1% phosphoric acid (H_3PO_4) in Milli-Q filtered water (RG Ultra-pure water system, Millipore Corp.), and solvent B) 0.1% H_3PO_4 in HPLC-grade acetonitrile. Data acquisition was applied for 45min with a total run of 65min. Gradient elution was as follows: 0% B at 0min, 0% B at 10min, 50% B at 50min, 95% B at 51min, 95% B at 56min, 0% B at 57min and 0%B at 65min. Flow rate was 1mL/min, except at 95%B, which was at 1.5mL/min. The detector was set at 280, 320, 370, and 520 nm. Phenolic compounds were identified by comparison of UV–visible spectra and retention times with the standards.

Sample preparation

Cabernet franc wine was filtered through 0.45μm nylon filter (Fisherbrand, PTFE, Fisher Scientific, Denver, CO) and directly injected for HPLC analysis using the chromatographic conditions described above.

Pomace AC extracts (5mL) were evaporated under N_2 flow in 35^{O}C water bath to remove the organic solvent. The aqueous extract left (≈2.5mL), was filtered through 0.45μm nylon filters and injected for HPLC analysis.

RESULTS AND DISCUSSION

Cabernet franc analyses

Initial analyses of Cabernet franc grapes for pH, soluble solids, and titratable acidity are shown below in Table 9. Sugar level was at 22%, with a pH at 4.58 and a titratable acidity at 3.57g/L. Beside the individual varietal difference, sugars, titratable acidity and pH are also dependent on climate and cultivation techniques. Our 22% sugar level of Cabernet franc, was lower than observed for Cabernet franc by some wineries (Brehm Vineyards, 2009; Trespass Vineyard, 2009), which ranged from 24-27% Brix. But it was equal or higher than the sugar content of 14-22% reported by other researchers (McCallum et al., 2009, Ryona et al., 2008, Zhang et al., 2007, Mazza et al., 1999). Our observed pH value (4.58) and the titratable acidity (3.57g/L) were higher and lower than other reports respectively, where pH ranged between 3.2 and 4.0 and titratable acidity

from 4.00-18.00g/L (McCallum et al., 2009, Ryona et al., 2008, Zhang et al., 2007, Mazza et al., 1999).

Table 9. Analyses of Cabernet franc grapes and wine.

	pH		Titratable Acidity (Tartaric Acid g/L)		Alcohol (% v/v)		Soluble Solids (%Brix)
Cabernet franc Grape juice	4.58		3.57				24.1
	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	
Wine Week1	4.27	4.30	5.73	5.68	12.5	13.1	
Wine Week15	3.90	3.88	5.92	6.00			

Tartaric acid was added on week11 during maturation to reduce the pH levels o wine.

Moisture content was performed on whole grape berries and pressed pomace, as well as on the ground pomace as described in materials and methods. Both Cabernet franc grapes and pomace had approximately 75% moisture (Table 10), which is close to the average moisture reported for grapes (81%) (University of Georgia, 2009; Botanical Online, 2009).

Table 10. Moisture content of Cabernet franc grapes and pomace.

	% Moisture	
Tissue	Rep1	Rep2
Whole grape berries	75.2	
Pomace	75.8	78.7
Ground pomace	73.9	76.6

Grape pomace accounts for about 13-20% of the total weight of grapes used in wine processing (Brenes et al. 2008, Ruberto et al. 2008). During our winemaking

process of Cabernet franc we produced approximately 25% pomace (Table 11), which is a little higher than what's reported, probably because we didn't press our grapes extensively and the final product had more moisture than a typical pomace pressed solely for wine. The weight distribution of Cabernet franc grapes during the wine processing is shown in Table 11 for the two replications. Both trials resulted in similar overall values in terms of % stems and yield for wine and pomace. Pomace, with about 75% moisture, accounts for only 5% of the initial grape weight as dry solid matter, which contains all the compounds under investigation.

Table 11. Cabernet franc grapes weight distribution of the winemaking process.

Processing Step	Fraction	Fraction weight (kg)		% recovery from whole grapes	
		Rep1	Rep2	Rep1	Rep2
	Cabernet franc Grapes	43.16	43.68	100	100
Desteming	Stems	2.31	2.47	5	6
	Must for fermentation	37.93	40.47	88	93
Pressing	Wine	24.36	25.24	56	58
	Pomace	9.45	11.69	22	27
	Dry pomace	2.28	2.49	5	6

Free and total SO_2 was monitored from Week1 through Week15 of wine stabilization as depicted in Figure 10 (p.55). The sulfur dioxide in wines is primarily used as a preservative to prevent oxidation and spoilage from microorganisms. Depending on

the time and levels added it may have different effects as well. Addition in grape juice will inhibit some of the enzymes that cause browning. When added at early fermentation steps it will control unhelpful yeasts that were present on the grapes. During fermentation it will inhibit the growth of bacteria, but should be avoided when malolactic fermentation is desired, due to the inhibitory action on the malolactic bacteria as well (Accuvin, 2009). Sulfur dioxide has three forms in water; molecular SO_2, sulfite (SO_3^{-2}) and bisulfite (HSO_3^{-1}). The most common forms in wine are molecular SO_2 and bisulfite (ETS Laboratories, 2009).

These two chemical forms are associated in water by Equation 2.

$$SO_2 + H_2O = HSO_3^{-1} + H^{+1} \qquad (2)$$

Free SO_2 procedure calculates both forms, but only the molecular SO_2 is effective against bacteria, which accounts only for 1-7% of the free. From equation (2) we may see the effect of acidity on SO_2 levels, whereas a more acidic matrix will favor the formation of molecular SO_2. A level at 0.8mg/L of molecular SO_2 is suggested as effective to prevent growth against spoilage bacteria, in combination with pH, alcohol content and temperature. For instance 40mg/L of free SO_2 at pH=3.5 or 125mg/L at pH=4.0 will have the equivalent 0.8mg/L of molecular SO_2. Addition and levels of SO_2 in Cabernet franc wines during maturation are shown below in table 12.

Table 12. SO_2 levels of Cabernet franc during 15 weeks of maturation.

		mg/L SO_2 (ppm)			
		Rep1		Rep2	
	Addition (ppm)	Free	Total	Free	Total
Week 1	50				
Week 4	50	3.2	18.4	2.4	12
Week 11	50	10.4	37.6	12	40
Week 15		24		29	

Four weeks after the initial addition of SO_2 in Cabernet franc wines, levels of free SO_2 were extremely low, therefore potassium metabisulfite was proportionally added to each replication in an effort elevate the free SO_2. Subsequent additions were performed in following weeks, after testing revealed that substantial amounts were bound in the wine. Probably the high pH of our wine, along with normal SO_2 loss, e.g. as gas or involved in chemical reactions with acetaldehydes and anthocyanins, didn't allow the additions of potassium metabisulfite to be in the free form (or molecular SO_2). Therefore addition of tartaric acid was performed on Week11 (Fig.10, p.55) to lower the pH from 4.3 (table 9, p.62) to 3.9 and increase the acidity. Testing of sulfites on Week15 showed that lowering the pH and addition of the same amount of potassium metabisulfite, increased the free SO_2 levels to 24mg/L (Table 12). This is still lower than the recommended level and suggests further monitoring and addition of SO_2 until bottling.

Antioxidant activity and total phenolics

Antioxidant activity was estimated for Cabernet franc pomace and wines using the ORAC assay as described in materials and methods. The best treatments of 50% Acetone (AC) and 70% Methanol (MT) from Experiment I were chosen for antioxidant extraction from the pomace.

Cabernet franc pomace

Both treatments of AC were significantly ($p<0.05$) more efficient in extracting antioxidants from Cabernet franc pomace than MT, a trend that was also true for Cynthiana extracts in Exp.I. However, Cabernet franc pomace showed higher antioxidant activity (55-83μmoles TE/g) than Cynthiana pomace (22-37μmoles TE/g) (Table 13), which is even greater if we account for the moisture content of Cabernet franc pomace as well. That would give a range of 220-332μmoles TE/g dry pomace for Cabernet franc and 48-74μmoles TE/g dry pomace for Cynthiana. In contrast, Hogan et al. (2009) observed no difference between the ORAC values of Norton and two clones of Cabernet franc, which was on average at ≈25μmoles TE/g pomace.

Table 13. Average ORAC of Cabernet franc and Cynthiana pomace.

			Average Activity (μmoles TE/gr tissue)	
			Cabernet franc	Cynthiana
	Shaking time (h)	% Moisture:	77.3	51.4
50% Acetone	8		83.8[a]	37.6[d]
	2		75.8[b]	36.1[d]
70% Methanol	8		56.3[c]	24.4[e]
	4		55.0[c]	22.6[e]

[a] Numbers with different letters denote significance at $p<0.05$.
All solvent treatments are 4:1 solvent to sample ratio

Duration of extraction had no difference in antioxidant recovery when MT was used, similar to Exp. I, however the AC treatments on Cabernet franc pomace showed significantly higher yield of antioxidants when extracted for 8h than 2h (Table 13). This comes in contrast with Exp. I, where no difference was observed between 8h and 2h of extraction for Cynthiana pomace.

Total phenolics and antioxidant activity were significantly positively correlated with r=0.96 (Table 14), and showed phenolic content for AC extracts of 2.44-2.78mg GAE/g pomace and for MT 1.04-1.25mg GAE/g pomace. Hogan et al. (2009) also reported average of 0.63 and 1.47mg GAE/g pomace for two different clones of Cabernet franc, which they extracted with 70% methanol as well.

Table 14. Antioxidant activity and total phenolics of Cabernet franc pomace extracts.

Treatment	Shaking time (h)	Average Activity (µmoles TE/gr tissue)		Total Phenolics (mg GAE/g tissue)		Pearson Correlation Coef. (r)
		Rep1	Rep2	Rep1	Rep2	
50% Acetone	8	86.6[a]	81.1[b]	2.78	2.75	0.96
	2	78.6[b]	73.1[c]	2.45	2.44	
70% Methanol	8	53.2[e]	59.3[d]	1.25	1.09	0.96
	4	58.9[d]	51.2[e]	1.16	1.15	

[a] **Numbers with different letters denote significance at p<0.05.**
All solvent treatments are 4:1 solvent to sample ratio

Difference of the antioxidant activities between the two replications was also observed. Total phenolics for raw grapes and pomace of Cabernet franc are shown in Table 15. Whole grapes had an average 5.25mg GAE/g tissue and pomace 5.36 and 3.93mg GAE/g tissue for Rep1 and Rep2 respectively.

Table 15. Total phenolic content of Cabernet franc grapes and pomace.

	Total Phenolics (mg GAE/g tissue)	
Tissue	**Rep1**	**Rep2**
Whole grape berries	5.25	
Pomace	5.36	3.93

Cabernet franc wine

Antioxidant activity and total phenolics of Cabernet franc wines were monitored for 15 weeks, with samples collected periodically (Fig. 7, p.60). Analyses was performed for Week1 and Week15, with Week1 being the first sampling after pressing. Antioxidant activity of Cabernet franc wine was on average 27.1μmoles TE/mL wine. An increase on ORAC values of Rep1 was observed for Week15 as opposed to Week1 (Table 16).

Table 16. Cabernet franc wine antioxidant activity and total phenolics.

	Average Activity (μmoles TE/mL wine)		Total Phenolics (mg GAE/L wine)		Pearson Correlation Coef. (r)
Time	**Rep1**	**Rep2**	**Rep1**	**Rep2**	
Week1	21.0[c]	29.2[ab]	702.0	764.5	0.89
Week15	27.6[b]	30.7[a]	794.3	790.1	

[a] Numbers with different letters denote significance at p<0.05.

Antioxidant activity of the wines was significantly higher in Rep2 than Rep1, which might be related to the distribution of antioxidants between pomace and wine,

0since the opposite effect was observed for the antioxidant activity of the pomace extracts (Rep1 showed higher values than Rep2). This may indicate a higher extraction of phenolics took place during fermentation of Rep 2. Total phenolics showed also a good positive correlation with antioxidant activity (r=0.89) with an average of 733.3 and 792.2 mg of GAE/L of wine in Week1 and Week15 respectively (Table 16). Anli and Vural (2009) reported ≈2300mg of GAE/L of C. sauvignon wines poduced in Turkey, and Salaha et al. (2008) 1900-2150 mg of GAE/L of C. sauvignon wines produced in Greece.

HPLC

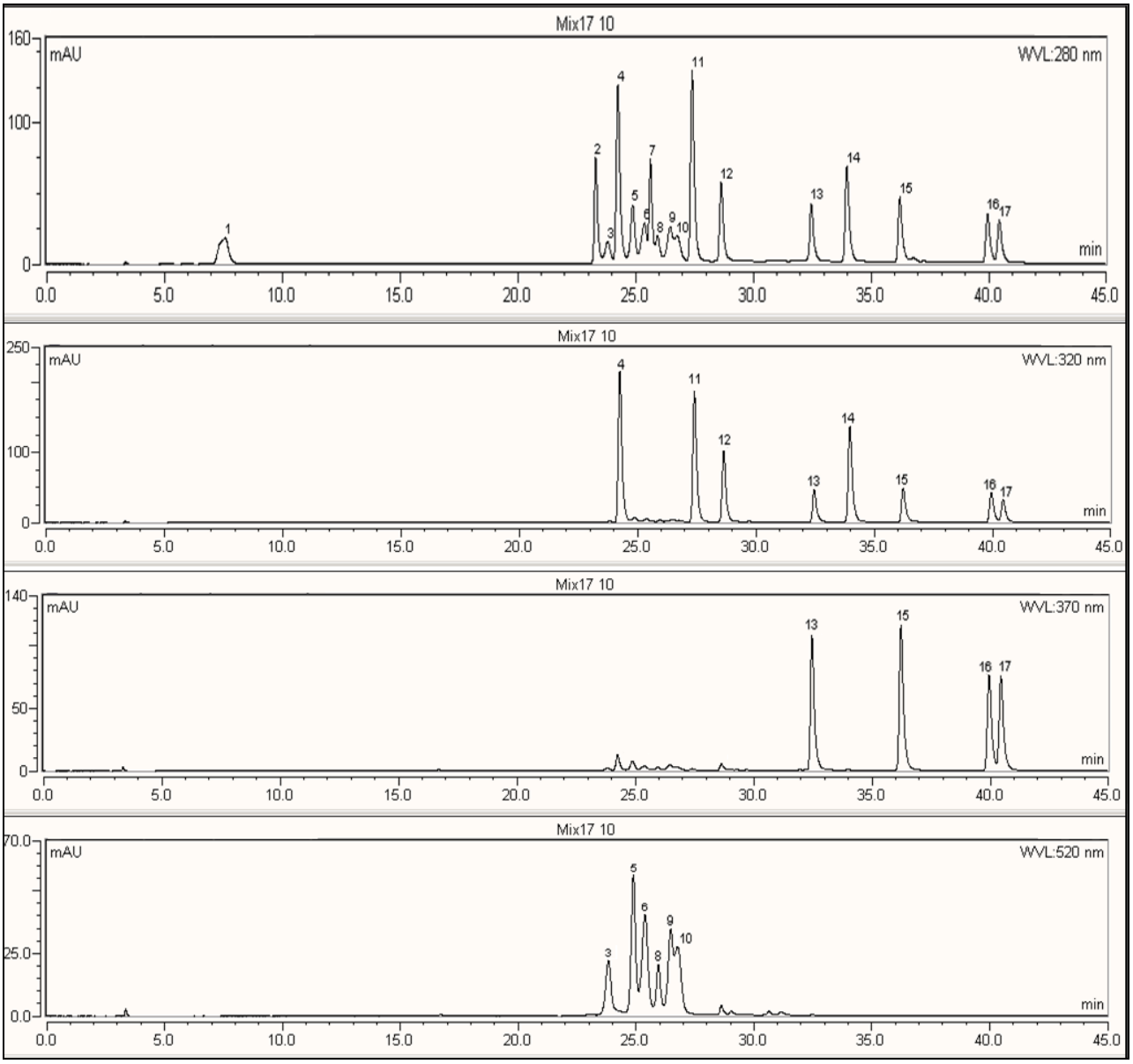
Cabernet franc pomace

The best treatment for antioxidant extraction of cabernet franc pomace (50% acetone, 8h, and 4:1 ratio) was analyzed in HPLC for major compound identification using the available standards mentioned above in the Materials and Methods section. Representative chromatograms for all the standards at all wavelengths used are shown below in Figure 9. Table 17 shows peak assignment and retention times for the standards. For identification of compounds in the sample we used the maximum absorbance for each set of polyphenols. In particular, for phenolic acids 280nm and 320nm were used, for anthoxanthins and stilbenes 280nm and 370nm, and for anthocyanins 520nm.

Table 17. Retention times of standards used for HPLC compound identification.

No.	Peakname	Ret.Time min
1	Gallic Acid	7.583
2	Catechin	23.317
3	Delphinidin 3-O-Glu	23.833
4	Caffeic	24.258
5	Cyanidin 3-O-Gluc	24.883
6	Petunidin 3-O-Gluc	25.383
7	Epicatechin	25.642
8	Pelargonidin 3-O-Gl	25.942
9	Peonidin 3-O_Gluc	26.475
10	Malvidin 3-O-Gluc	26.767
11	Coumaric	27.408
12	Ferrulic	28.642
13	Myricetin	32.467
14	Resveratrol	33.975
15	Quercetin	36.225
16	Kaempherol	39.950
17	Isorhamnetin	40.450

Figure 9. HPLC chromatograms for polyphenolic standards at 280, 320, 370, and 520nm.

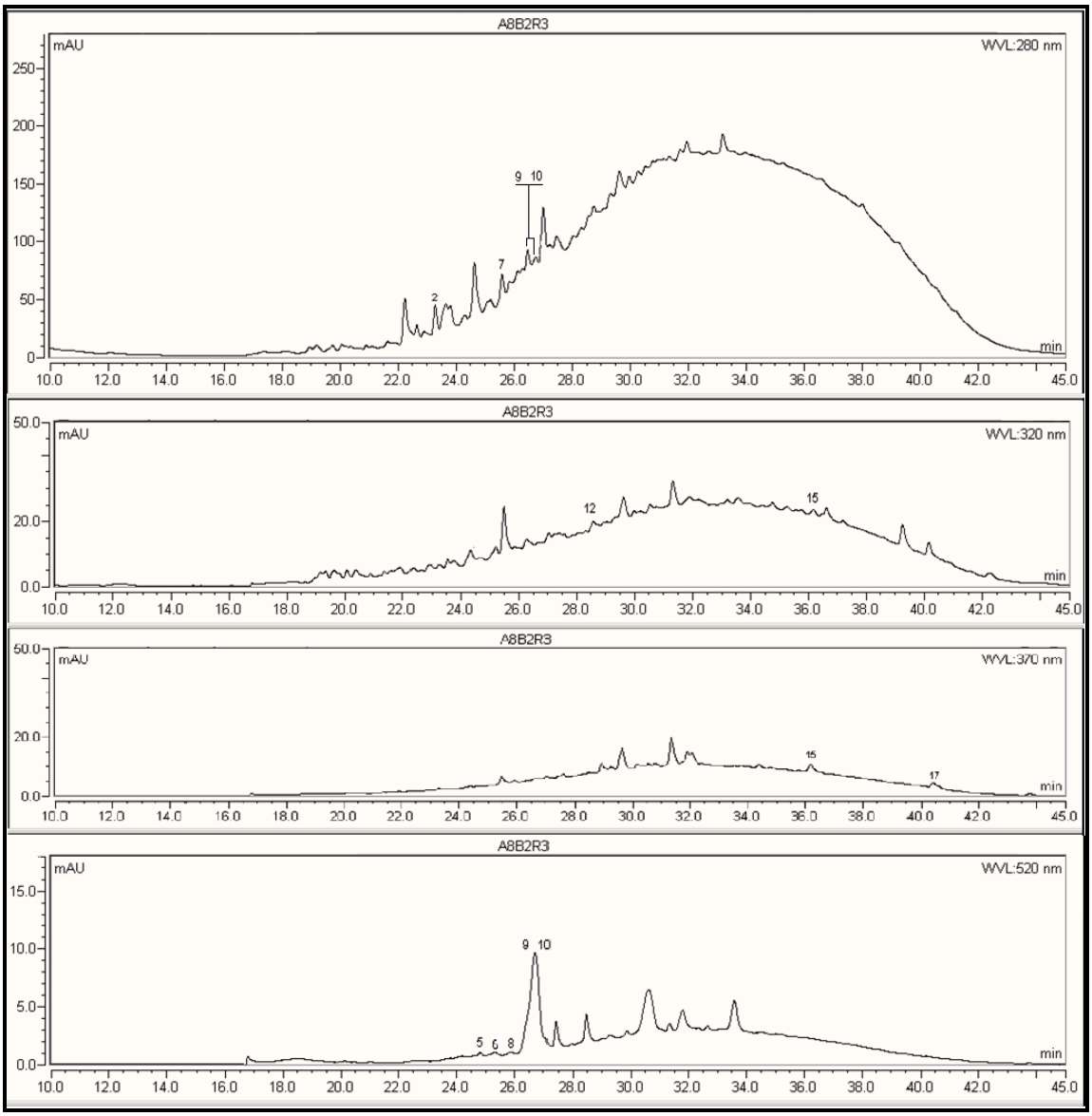


Peak assignment: 1. Gallic acid, 2. Catechin, 3. Delphinidin-3-O-glucoside, 4. Caffeic acid, 5. Cyanidin-3-O-glucoside, 6. Petunidin-3-O-glucoside, 7. Epicatechin, 8. Pelargonidin-3-O-glucoside, 9. Peonidin-3-O-glucoside, 10. Malvidin-3-O-glucoside, 11. Coumaric acid, 12. Ferrulic acid, 13. Myricetin, 14. Resveratrol, 15. Quercetin, 16. Kaempherol and 17. Isorhamnetin.

With HPLC analysis of Cabernet franc pomace, catechin and epicatechin were the major compounds identified with the available standards, while ferrulic acid, quercetin, isorhamnetin, cyanidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, and pelargonidin 3-*O*-glucoside were also detected. Peonidin 3-*O*-glucoside and Malvidin 3-*O*-glucoside had

adjacent retention times with the gradient used, thus a major peak appearing at 520nm is more likely a combination of the two compounds, with malvidin 3-*O*-glucoside probably being the predominant compound of the two since malvidin has been extensively identified as the major anthocyanin in red grapes (Amico et al. 2008, Nicoletti et al. 2008, Cho et al. 2004) (Figure 10).

Figure 10. HPLC chromatograms of Cabernet franc pomace extracts at 280, 320, 370, and 520nm.



Peaks identified by retention time of available standards (Table 17) include: 2. Catechin, 5. Cyanidin 3-*O*-glucoside, 6. Petunidin 3-*O*-glucoside, 7. Epicatechin, 8.Pelargonidin 3-*O*-glucoside, 9. Peonidin 3-*O*-glucoside, 10. Malvidin 3-*O*-glucoside, 12. Ferrulic acid, 15. Quercetin, 17. Isorhamnetin.

Thimothe et al. (2007) analyzed also C. franc pomace in HPLC and found malvidin in greater amounts than peonidin. Similar to our C. franc, their major compounds identified were also predominately catechin followed by malvidin, cyanidin and delphinidin. Kammerer et al. (2004) had also identified malvidin 3-*O*-glucoside as the major anthocyanin in 5 red grape pomace extracts.

Pomace is also rich in seeds, which are also likely to release a number of phenolic compounds during grinding at sample preparation. Guendez et al. (2005) investigated low molecular polyphenolics in grape seed extracts of mostly red varieties (including Cabernet sauvignon) and found catechin and epicatechin being the major compounds identified.

Cabernet franc wine

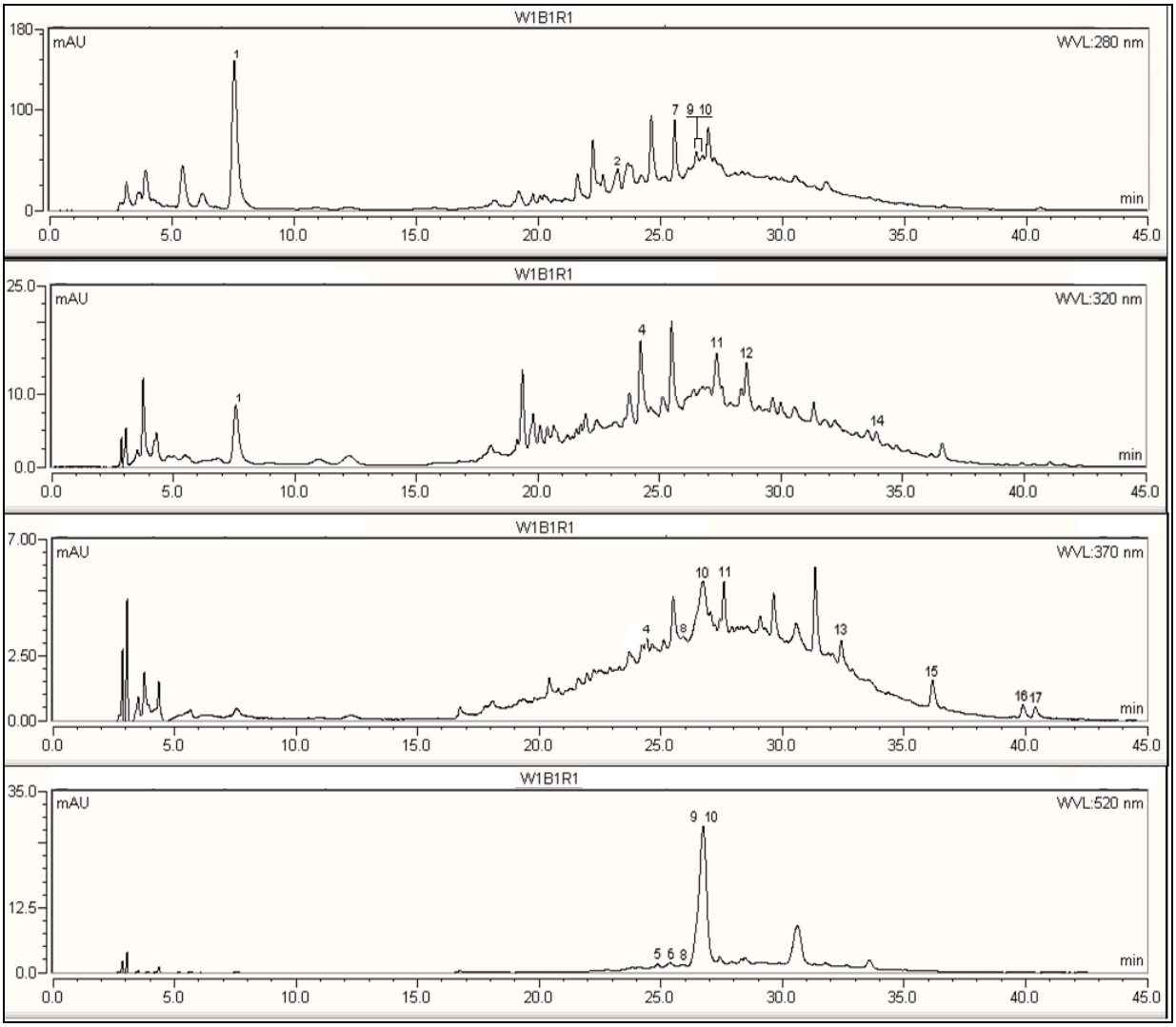
Wine from Cabernet franc was analyzed with HPLC as described in the Materials and Methods section above. Results showed that the major compounds in Cabernet franc were predominately gallic acid and epicatechin. Anli and Vural (2009) tested red wines of Turkey (including Cabernet sauvignon) for phenolic substances and also found gallic acid, catechin and epicatechin being the major compounds present.

Similarly to pomace, peonidin and malvidin 3-*O*-glucoside probably contributed to one major peak at 520nm. However, based on the work of previous researchers, we believe that malvidin is probably responsible for this response. Kallithraka et al. (2005) analyzed 17 red grape varieties cultivated in Greece, including the closely related Cabernet sauvignon, for anthocyanin composition and all of them contained malvidin 3-

O-glucoside as the major component by several -fold times. Similar results were obtained by Salaha et al. (2008).

Smaller amounts of caffeic, coumaric and ferrulic acids were also identified, along with traces of myricetin, quercetin, kaempherol and isorhamnetin (Figure 11). A few other compounds with their major absorbance at 280nm were not identified.

Figure 11. HPLC chromatograms of Cabernet franc wine at 280, 320, 370, and 520nm.



Peaks identified by retention time of available standards (Table 17) include: 1. Gallic acid, 2. Catechin, 4. Caffeic acid, 5. Cyanidin 3-*O*-glucoside, 6. Petunidin 3-*O*-glucoside, 7. Epicatechin, 8. Pelargonidin 3-*O*-glucoside, 9. Peonidin 3-*O*-glucoside, 10. Malvidin 3-*O*-glucoside, 11. Coumaric acid, 12. Ferrulic acid, 13. Myricetin, 15. Quercetin, 16. Kaempherol, 17. Isorhamnetin.

CONCLUSIONS

Cabernet franc wine and pomace showed high antioxidant activity of approximately 27μmoles TE/mL wine and 56-83μmoles TE/g pomace respectively. This suggests that high-value compounds may still be present in significant amounts in the Cabernet franc pomace waste materials. Antioxidant activity was positively correlated with TPC, both for wine and pomace, with average of 762mg GAE/L of wine and 2mg GAE/g pomace respectively. The major compounds identified in wine with HPLC were predominately gallic acid and epicatechin, with malvidin 3-*O*-glucoside in smaller amounts. Pomace extracts contained mainly catechin and epicatechin, with malvidin 3-*O*-glucoside as the major anthocyanin. Several major peaks were not identified, which suggests that more compounds might also be responsible for the antioxidant properties of Cabernet franc. Both Cabernet franc pomace and wine proved to have good antioxidant activities, thus being good sources of valuable natural biomolecules.

REFERENCES

Accuvin -- How SO2 and pH are Linked. 2009. http://www.accuvin.com/pHSO2Links.pdf 17 Novermber, 2009).

Anli, R.E. and N. Vural. 2009. Antioxidant Phenolic Substances of Turkish Red Wines from Different Wine Regions. Molecules. 14 289-297.

Botanical Online – Properties of Grapes. 2009. http://www.botanical-online.com/uvasangles.htm (17 November, 2009).

Brehm Vineyards – varietal information. 2009. http://www.brehmvineyards.com/ (17 November, 2009).

Brenes, A., S.G. S Lyago-Ayerdy, I. Arija, F. Saura-Calixto, A. Viveros, I. Go li and C. Centeno. 2008. Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens. Poultry science. 87(2):307-316.

Cho, M.J., J.R. Clark, R.L. Prior and L.R. Howard. 2004. Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. Journal of the Science of Food and Agriculture. 84 1771-1782.

ETS Laboratories -- Technical Bulletin - Molecular Sulfur Dioxide. 2009. http://www.etslabs.com/resources/publications/molecular-sulfur-dioxide/technical-bulletin.aspx (17 November, 2009).

Guendez, R., S. Kallithraka, D.P. Makris and P. Kefalas. 2005. Determination of low molecular weight polyphenolic constituents in grape (*Vitis vinifera* sp.) seed extracts: Correlation with antiradical activity. Food chemistry. 89 1-9.

Hogan, S., B. Zoecklein, K. Zhou, L. Zhang and J. Li. 2009. Antioxidant properties and bioactive components of Norton (*Vitis aestivalis*) and Cabernet Franc (Vitis vinifera) wine grapes. Food science and technology. 42 1269-1274.

Kammerer, D., A. Claus, R. Carle and A. Schieber. 2004. Polyphenol screening of pomace from red and white grape varieties (Vitis vinifera L.) by HPLC-DAD-MS/MS. Journal of agricultural and food chemistry. 52 (14):4360-4367.

Mazza, G., L. Fukumoto, P. Delaquis, B. Girard and B. Ewert. 1999. Anthocyanins, phenolics, and color of Cabernet Franc, Merlot, and Pinot Noir wines from British Columbia. Journal of agricultural and food chemistry. 47 4009-4017.

McCallum, J.L., R. Tsao, J.N. Strommer and H. Fisher. 2009. Effect of Terroir on anthocyanins of Vitis vinifera L. 'Cabernet Sauvignon' and 'Cabernet Franc' grown in different Niagara appellations. Acta horticulturae. 287-294.

Nicoletti, I., D. Corradini, A. De Rossi and C. Bello. 2008. Identification and Quantification of Phenolic Compounds in Grapes by HPLC-PDA-ESI-MS on a Semimicro Separation Scale. Journal of agricultural and food chemistry. 56 (19):8801-8808.

Ruberto, G., A. Renda, V. Amico and C. Tringali. 2008. Volatile components of grape pomaces from different cultivars of Sicilian vitis vinifera l. Bioresource technology. 99(2):260-268.

Ryona, I., A.N. Lakso, G.L. Sacks, B.S. Pan and D.S. Intrigliolo. 2008. Effects of Cluster Light Exposure on 3-Isobutyl-2-methoxypyrazine Accumulation and Degradation Patterns in Red Wine Grapes (Vitis vinifera L. Cv. Cabernet Franc). Journal of agricultural and food chemistry. 56 (22):10838-10846.

Salaha, M.-I., E. Koussissi, I. Tzourou, S. Kallithraka and I. Marmaras. 2008. A natural alternative to sulphur dioxide for red wine production: Influence on colour, antioxidant activity and anthocyanin content. Journal of food composition and analysis. 21 660-666.

Thimothe, J., I.A. Bonsi, O.I. Padilla-Zakour and H. Koo. 2007. Chemical Characterization of Red Wine Grape (*Vitis vinifera* and *Vitis* Interspecific Hybrids) and Pomace Phenolic Extracts and Their Biological Activity against *Streptococcus mutans*. Journal of agricultural and food chemistry. 55 (25):10200-10207.

Trespass Vineyard – 2005 Cabernet Franc. 2009. http://www.trespassvineyard.com/wines/cabernet-franc-05.html (17 November, 2009).

University of Georgia – Grapes, *Vitis* spp. 2009. http://www.uga.edu/fruit/grape.html (17 November, 2009).

Zhang, M., Q. Xu, C. Duan, W. Qu and Y. Wu. 2007. Comparative study of aromatic compounds in young red wines from cabernet sauvignon, cabernet franc, and cabernet gernischet varieties in China. Journal of Food Science. 72 (5):C248-52.

CHAPTER V

CONCLUSIONS

Antioxidant and natural compounds have been increasingly of interest and in demand during the last decade. Industrial fruit and vegetable processing, which generates large amounts of waste, may contain valuable byproducts. Our research focused on screening wine industry waste for antioxidants and developing a relatively easy method for extraction, which may be used with bulk volumes in actual industrial scale processing.

Cynthiana pomace was used as a model for the initial development of a rapid, single-solvent, and scalable extraction protocol. Out of the 32 solvent treatments, 50% Acetone/Water mix proved to be a good extraction medium when used for 2h at 2:1 solvent:sample ratio. Yield of 31μmoles TE/g tissue suggest that Cynthiana pomace is a good source of natural antioxidants, with anthocyanins cyanidin 3-*O*-glucoside and malvidin 3-*O*-glucoside being two of the major compounds in the extracts.

Cabernet franc grapes were also evaluated for antioxidant capacity at several stages of processing during a small scale wine production process. The pomace produced was extracted with the protocol developed using Cynthiana pomace, and the wine was tested for antioxidants over a period of 15 weeks. Both wine and pomace showed high antioxidant activity of approximately 27μmoles TE/mL wine and 56-83μmoles TE/g

pomace respectively. Antioxidant activity was also positively correlated with total phenolic content, both for wine and pomace, with average of 762mg Gallic acid equivalents/L of wine and 2mg Gallic acid equivalents/g pomace respectively. The major compounds identified in wine with HPLC were predominately gallic acid and epicatechin, with malvidin 3-*O*-glucoside in smaller amounts. Pomace extracts contained mainly catechin and epicatechin, with malvidin 3-*O*-glucoside as the major anthocyanin. Several major peaks were not identified, which suggests that more compounds might also be responsible for the antioxidant properties of Cabernet franc.

Both experiments suggest that grape pomace from red grape varieties typically retains significant amounts of antioxidant compound after winemaking. Our experiments also suggest that these compounds may be extracted using a relatively simple and scalable extraction process. Thus, red grape pomace may typically constitute a high-value waste stream, one from which wineries may be able to recover significant value in the form of natural antioxidant compounds. Quantification and identification of all compounds present in the pomace is needed in order to give us a better understanding of the true value of these winery waste streams. They will also help to elucidate the economics of extractions and such further processing as may be required to produce functional ingredients.

Beyond the direct evaluation of red grape pomace, our experiments served to develop and demonstrate a potentially industrial scale single-solvent extraction process for the screening of antioxidant compounds from grape pomace and possibly other agricultural waste streams as well.

Original from Duddoné et al. 2009

Table 1. Radical Scavenging Capacity of 30 Aqueous Plant Extracts[a]

plant	part of plant	DPPH inhibition %	ABTS inhibition %	ORAC (μmol Trolox/g)
Abelmoschus moschatus	seed	2.30 ± 0.59	1.48 ± 2.02	213 ± 4
Actinidia chinensis	flower	2.29 ± 0.33	2.12 ± 1.56	887 ± 56
Cananga odorata	flower	3.57 ± 0.16	4.13 ± 1.04	560 ± 10
Carica papaya	leaf	1.22 ± 1.02	1.38 ± 0.46	348 ± 17
Ceratonia siliqua	pod	7.70 ± 1.00	9.75 ± 0.56	225 ± 11
Cinnamomum zeylanicum	bark	84.43 ± 3.48	64.88 ± 3.74	8515 ± 300
Cistus ladaniferus	leaf	5,06 ± 1.03	26.83 ± 1.96	1410 ± 53
Coffea arabica	seed	41.21 ± 0.08	26.45 ± 0.22	3511 ± 57
Daucus carota	seed	1.22 ± 0.24	2.68 ± 1.02	435 ± 16
Eucalyptus globulus	leaf	27.43 ± 0.35	41.14 ± 0.51	2846 ± 134
Eugenia caryophyllus clovis	flower -bud	31.58 ± 4.73	46.68 ± 0.73	3084 ± 65
Ilex paraguariensis	leaf	71.75 ± 1.22	32.73 ± 3.51	5092 ± 314
Jasminum grandiflorum	flower	14.35 ± 4.65	10.20 ± 0.98	2330 ± 64
Juniperus communis	fruit	1.92 ± 1.81	0.97 ± 0.94	183 ± 18
Laurus nobilis	leaf	18.93 ± 1.20	18.61 ± 0.44	2963 ± 35
Lavandula augustifolia	flower	1.46 ± 0.25	2.38 ± 0.17	697 ± 27
Lavandula hybrida grosso	flower	2.84 ± 0.17	8.32 ± 0.08	1181 ± 28
Liriodendron tulipifera	leaf	3.99 ± 0.08	9.99 ± 0.2	1146 ± 37
Matricaria recutita	flower	0.67 ± 0.38	5.97 ± 0.16	588 ± 29
Myrocarpus fastigiatus	wood	39.73 ± 0.14	29.68 ± 0.65	5422 ± 78
Pinus maritima	bark	94.51 ± 0.01	76.71 ± 0.37	6506 ± 120
Pinus maritima (commercial extract)	bark	92.79 ± 0.69	83.68 ± 0.80	7727 ± 135
Populus nigra	bud	19.82 ± 2.28	16.76 ± 0.07	2738 ± 43
Quercus robur	wood	88.60 ± 2.04	99.80 ± 0.07	3850 ± 121
Ribes nigrum	bud	7.35 ± 0.58	21.87 ± 7.24	1138 ± 26
Rosa damascena	flower	36.95 ± 2.30	30.01 ± 1.18	2382 ± 62
Salvia sclarea	herb	0.19 ± 0.08	0.15 ± 0.26	330 ± 8
Styrax benjoin	resin	8.10 ± 0.30	27.79 ± 0.06	3635 ± 18
Trigonella foenum graecum	seed	9.23 ± 0.66	13.27 ± 0.62	4114 ± 132
Vanilla planifolia	pod	0.89 ± 0.29	2.56 ± 0.18	1593 ± 12
Zingiber officinalis	root	0.25 ± 0.94	3.14 ± 0.44	370 ± 28

[a] Data are expressed as the mean of triplicate ± SD.

Table 2. Superoxide Radical Scavenging Capacity, Ferric Reducing Capacity, and Total Phenolic Content of 30 Aqueous Plant Extracts[a]

plant	part of plant	SOD inhibition %	FRAP (mmol Fe^{2+}/g)	total phenolics (mg GAE/g)
Abelmoschus moschatus	seed	1.65 ± 0.003	0.08 ± 0.01	14.84 ± 0.17
Actinidia chinensis	flower	0.46 ± 0.008	0.40 ± 0.02	37.48 ± 0.23
Cananga odorata	flower	5.77 ± 0.017	0.37 ± 0.02	26.03 ± 1.16
Carica papaya	leaf	0.73 ± 0.006	0.55 ± 0.01	31.76 ± 0.62
Ceratonia siliqua	pod	11.61 ± 0.040	0.68 ± 0.01	23.58 ± 0.01
Cinnamomum zeylanicum	bark	51.79 ± 0.014	6.48 ± 0.15	309.23 ± 0.05
Cistus ladaniferus	leaf	33.72 ± 0.013	3.02 ± 0.07	103.21 ± 0.43
Coffea arabica	seed	49.83 ± 0.037	2.73 ± 0.03	173.49 ± 1.86
Daucus carota	seed	1.65 ± 0.006	0.31 ± 0.01	20.08 ± 0.11
Eucalyptus globulus	leaf	49.79 ± 0.051	4.66 ± 0.06	113.68 ± 0.33
Eugenia caryophyllus clovis	flower -bud	51.75 ± 0.023	7.00 ± 0.13	212.85 ± 2.96
Ilex paraguariensis	leaf	52.44 ± 0.010	4.67 ± 0.08	202.60 ± 5.16
Jasminum grandiflorum	flower	7.96 ± 0.030	0.89 ± 0.01	86.71 ± 1.11
Juniperus communis	fruit	0.54 ± 0.009	0.24 ± 0.09	6.86 ± 0.11
Laurus nobilis	leaf	17.88 ± 0.023	1.54 ± 0.01	59.85 ± 0.23
Lavandula augustifolia	flower	0.15 ± 0.001	0.14 ± 0.02	27.42 ± 0.41
Lavandula hybrida grosso	flower	4.54 ± 0.002	0.43 ± 0.01	55.11 ± 1.04
Liriodendron tulipiferum	leaf	11.92 ± 0.007	0.63 ± 0.03	53.04 ± 1.11
Matricaria recutita	flower	3.50 ± 0.001	0.12 ± 0.01	33.83 ± 0.75
Myrocarpus fastigiatus	wood	58.59 ± 0.021	2.34 ± 0.13	119.14 ± 1.58
Pinus maritima	bark	53.48 ± 0.034	6.45 ± 0.15	360.76 ± 0.04
Pinus maritima (commercial extract)	bark	60.32 ± 0.019	7.33 ± 0.06	363.02 ± 0.02
Populus nigra	bud	19.68 ± 0.009	2.10 ± 0.03	104.45 ± 0.69
Quercus robur	wood	81.20 ± 0.007	15.92 ± 0.17	397.03 ± 0.05
Ribes nigrum	bud	12.34 ± 0.026	1.75 ± 0.01	76.80 ± 0.39
Rosa damascena	flower	42.10 ± 0.032	5.08 ± 0.07	124.86 ± 1.54
Salvia sclarea	herb	2.35 ± 0.001	0.17 ± 0.01	17.56 ± 0.24
Styrax benjoin	resin	14.46 ± 0.032	3.08 ± 0.07	145.47 ± 1.76
Trigonella foenum graecum	seed	14.38 ± 0.034	2.18 ± 0.02	104.79 ± 1.83
Vanilla planifolia	pod	1.77 ± 0.006	0.97 ± 0.09	51.64 ± 0.35
Zingiber officinalis	root	3.00 ± 0.015	0.28 ± 0.01	26.18 ± 0.23

[a] Data are expressed as the mean of triplicate ± SD.

Original from Bellido and Beta 2009

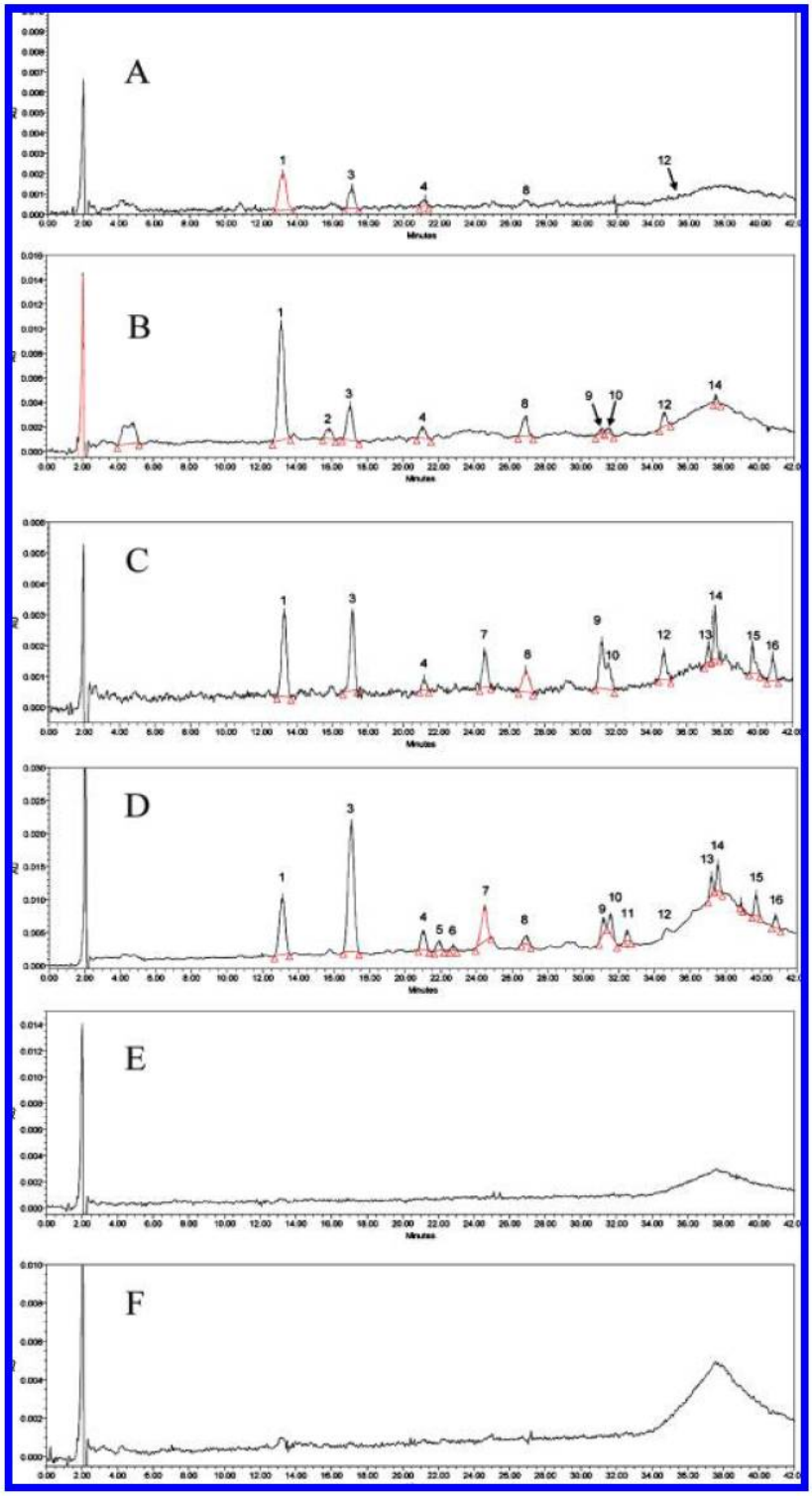


Figure 4. Typical illustration of HPLC chromatograms (520 nm) of anthocyanins obtained from 100% kernel flour (**A**, **C**, **E**) and the bran (**B**, **D**, **F**) of yellow, purple, and black barley, respectively. The number above each peak corresponds to the anthocyanin compound numbers in **Table 2**.

Table 2. Anthocyanin Composition[a,b] (Micrograms per Gram of Dry Weight) of Two Barley Genotypes Ground into Flour (Yellow and Purple) or Pearled To Remove 10% of Their Outer Bran Layers (Yellow Bran and Black Bran)

compd	RT (min)	anthocyanin[c]	yellow	yellow bran	purple	purple bran	LSD[d]
1	13.2	Dp-3-glc	104 ± 71 b	737 ± 232 a	93 ± 60 b	290 ± 252 b	335
2	15.9	Dp-3-rut	ND	87 ± 10	ND	ND	
3	17.1	Cy-3-glc	30 ± 15 c	177 ± 97 b	99 ± 42 bc	505 ± 174 a	141
4	21.1	Pt-3-glc	20 ± 9 c	73 ± 11 b	37 ± 24 c	118 ± 17 a	25
5	22.0	unknown	ND	ND	ND	40 ± 14	
6	22.8	unknown	ND	ND	ND	28 ± 16	
8	24.6	unknown	ND	ND	50 ± 17 b	261 ± 88 a	119
9	26.9	unknown	47 ± 23 b	320 ± 280 a	44 ± 16 b	405 ± 204 a	328
10	31.2	unknown	ND	16 ± 7 b	79 ± 59 ab	100 ± 42 a	79
11	31.6	unknown	ND	52 ± 34 b	26 ± 12 b	607 ± 303 a	332
12	32.5	Cy-Cl	ND	ND	ND	9 ± 3	
13	34.7	unknown	9 ± 6 b	99 ± 80 ab	32 ± 17 ab	102 ± 49 a	90
14	37.2	unknown	ND	ND	19 ± 2 b	521 ± 263 a	351
15	37.6	unknown	ND	26 ± 4 b	37 ± 19 b	83 ± 27 a	30
16	39.7	unknown	ND	ND	30 ± 13 b	305 ± 147 a	197
17	40.8	unknown	ND	ND	27 ± 11 b	160 ± 76 a	102
			total	1587 ± 85 b	573 ± 264 bc	3534 ± 1244 a	1202

[a] Mean ± SD. [b] Moisture content of samples was as follows: 8.36% (yellow), 7.27% (yellow bran), 8.92% (purple), and 7.24% (purple bran). [c] Cy, cyanidin; Dp, delphinidin; Pt, petunidin; glc, glucoside; rut, rutinoside. Unknown anthocyanins were quantified in terms of cyanidin 3-glucoside equivalents. [d] LSD denotes Fisher's least significant difference. Means within each row followed by the same letter are not significantly ($P < 0.05$) different.

Table 3. ORAC[a] Values for the Methanolic Extracts Obtained from the Experimental Barley Samples

	mean[b] ($n = 3$) ORAC value (LSD = 753)
whole ground	
yellow	5601 ± 329 d
purple	3937 ± 223 e
black	5430 ± 438 d
10% outer bran	
yellow bran	9004 ± 411 c
purple bran	11157 ± 375 a
black bran	10254 ± 643 b

[a] Expressed as micromoles of Trolox equivalents per 100 g of sample, on a dry weight basis. [b] Means within each column followed by the same letter are not significantly ($p < 0.05$) different.

Original from Oh et al. 2008

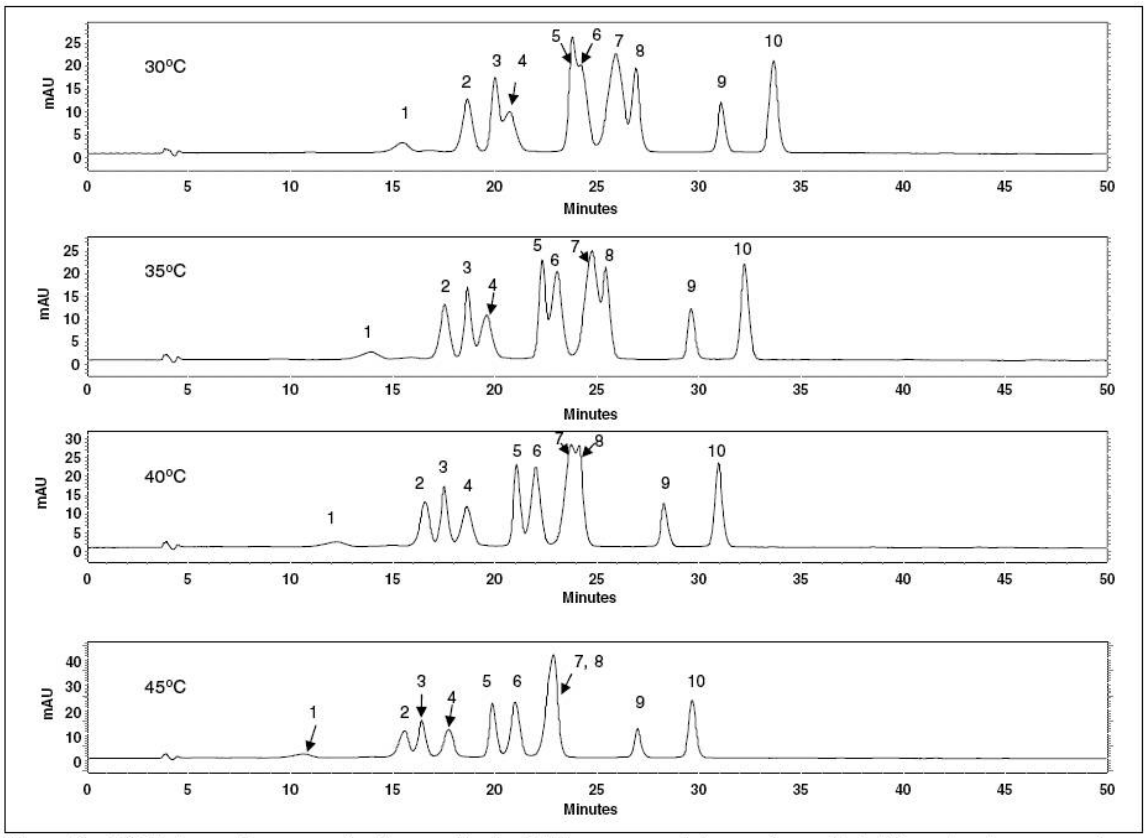


Figure 3—HPLC chromatograms of anthocyanins in Oil-Meoru grape juice performed at different column oven temperature.

Original from Guerrero et al. 2008

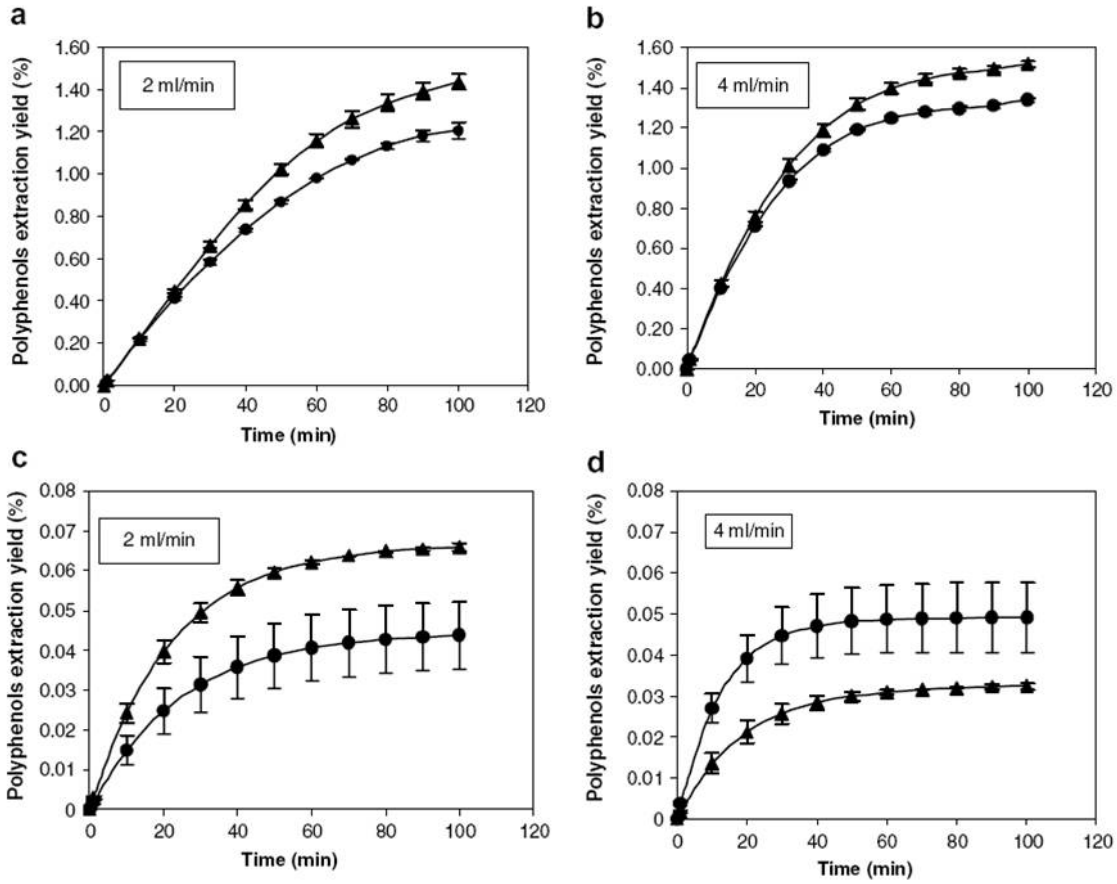


Fig. 1. Evolution of polyphenols extraction yield. (a) water, 2 ml/min; (b) water, 4 ml/min; (c) ethanol, 2 ml/min and (d) ethanol, 4 ml/min. (●: 40 °C; ▲: 50 °C).

Original from Kammerer et al. 2004

Table 1. LC-MS Data of Phenolic Compounds[a] Extracted from Grape Pomace (*V. vinifera* L.)

no.	compound	retention time (min)	$[M]^+$ $[M-H]^-$ *m/z*[b]	MS/MS fragments *m/z*[b]
	Anthocyanins			
1	delphinidin 3-*O*-glucoside	9.7	465	303
2	cyanidin 3-*O*-glucoside	11.8	449	287
3	petunidin 3-*O*-glucoside	13.2	479	317
4	peonidin 3-*O*-glucoside	15.6	463	301
5	malvidin 3-*O*-glucoside	16.8	493	331
6	delphinidin 3-*O*-acetylglucoside	18.2	507	303
7	petunidin 3-*O*-acetylglucoside	22.3	521	317
8	peonidin 3-*O*-acetylglucoside	24.6	505	301
9	malvidin 3-*O*-acetylglucoside	25.8	535	331
10	cyanidin 3-*O*-*p*-coumaroylglucoside	27.0	595	287
11	petunidin 3-*O*-*p*-coumaroylglucoside	28.1	625	317
12	peonidin 3-*O*-*p*-coumaroylglucoside	31.4	609	301
13	malvidin 3-*O*-*p*-coumaroylglucoside	32.3	639	331
	Phenolic Acids[a]			
14	gallic acid	5.9	169	125
15	5-(hydroxymethyl)furfural	7.7		
16	protocatechuic acid	12.7	153	109
17	caftaric acid	13.8	311	179
18	*p*-hydroxybenzoic acid	18.0	137	93
19	coutaric acid	20.8	295	163
20	caffeic acid	23.4	179	135
21	fertaric acid	24.3	325	193
22	syringic acid	25.7	197	153/182
23	*p*-coumaric acid	31.0	163	119
24	sinapic acid	32.1	223	164/208
25	ferulic acid	32.5	193	134
	Anthoxanthins and Stilbenes			
26	procyanidin B1	13.7	577	407/425
27	catechin	17.5	289	245
28	procyanidin B2	21.4	577	407/425
29	epicatechin	25.4	289	245
30	epicatechin gallate	41.9	441	289
31	*trans*-polydatin	42.5	389	227
32	quercetin 3-*O*-galactoside	49.5	463	301
33	quercetin 3-*O*-glucoside	50.4	463	301
34	quercetin 3-*O*-glucuronide	50.9	477	301
35	quercetin 3-*O*-rhamnoside	53.4	447	301
36	kaempferol 3-*O*-glucoside	56.4	447	284/285
37	isorhamnetin 3-*O*-glucoside	57.4	477	314/315
38	*trans*-resveratrol	59.2	227	185
39	quercetin	66.2	301	151/179
40	kaempferol	71.1	285	257

[a] And 5-(hydroxymethyl)furfural. [b] Positive ion mode, anthocyanins; negative ion mode, phenolic acids, anthoxanthins, and stilbenes.

Original from Kammerer et al. 2004

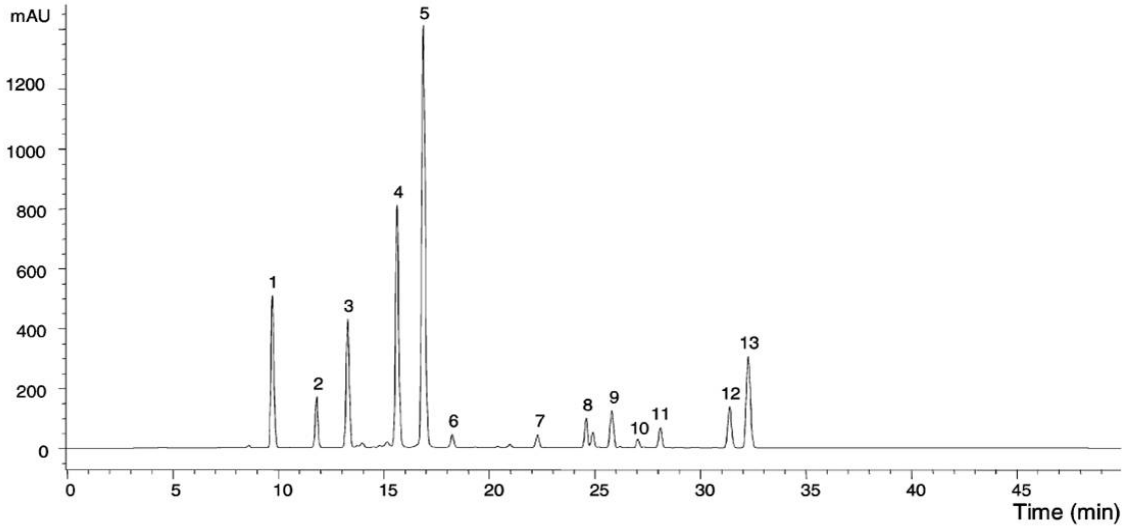


Figure 1. HPLC separation of anthocyanins from a Cabernet Mitos extract (520 nm). For peak assignment see **Table 1**.

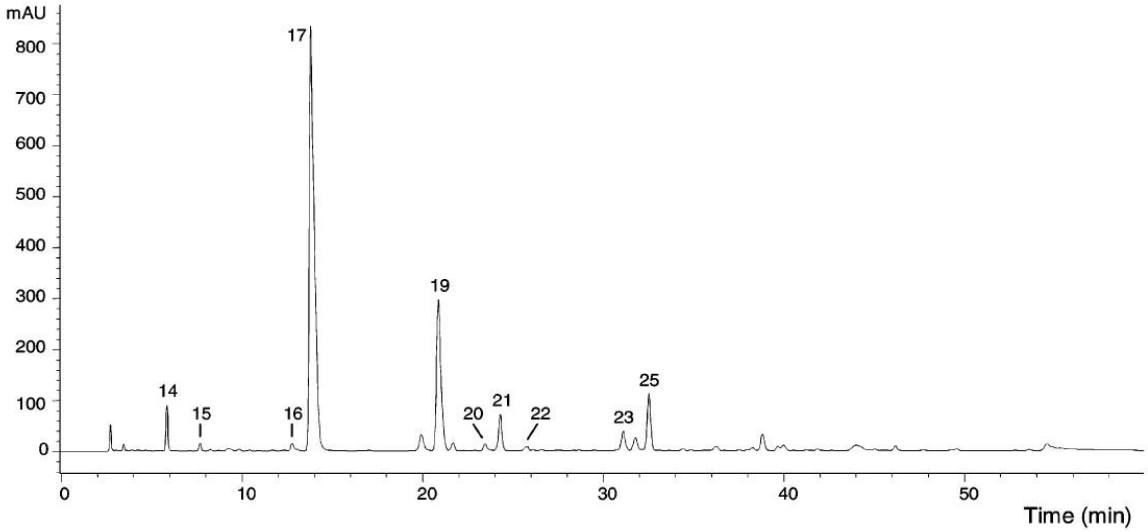


Figure 2. HPLC separation of phenolic acids from a Cabernet Mitos extract (280 nm). For peak assignment see **Table 1**.

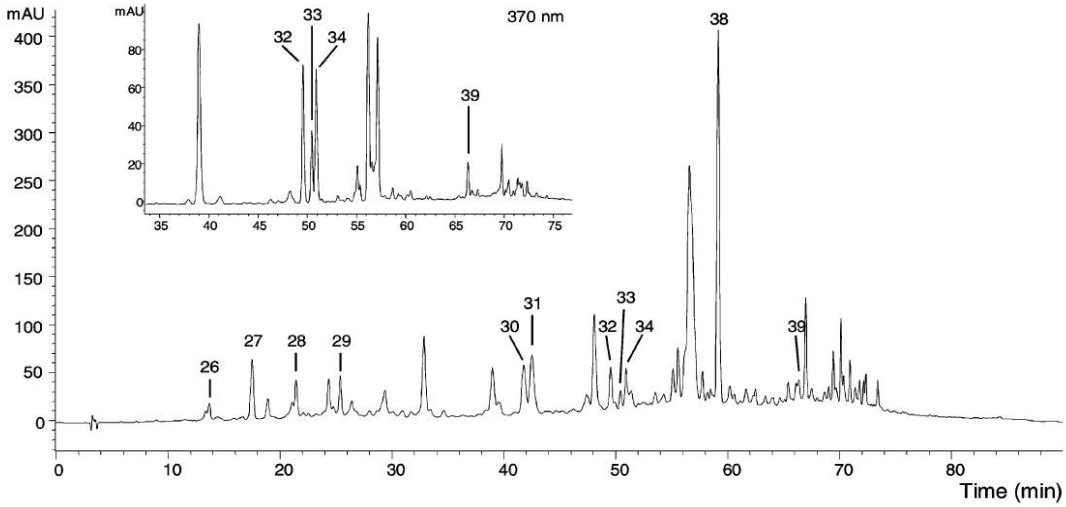


Figure 3. HPLC separation of anthoxanthins and stilbenes from a Cabernet Mitos extract (280 and 370 nm). For peak assignment see **Table 1**.

Original from Thimothe et al. 2007

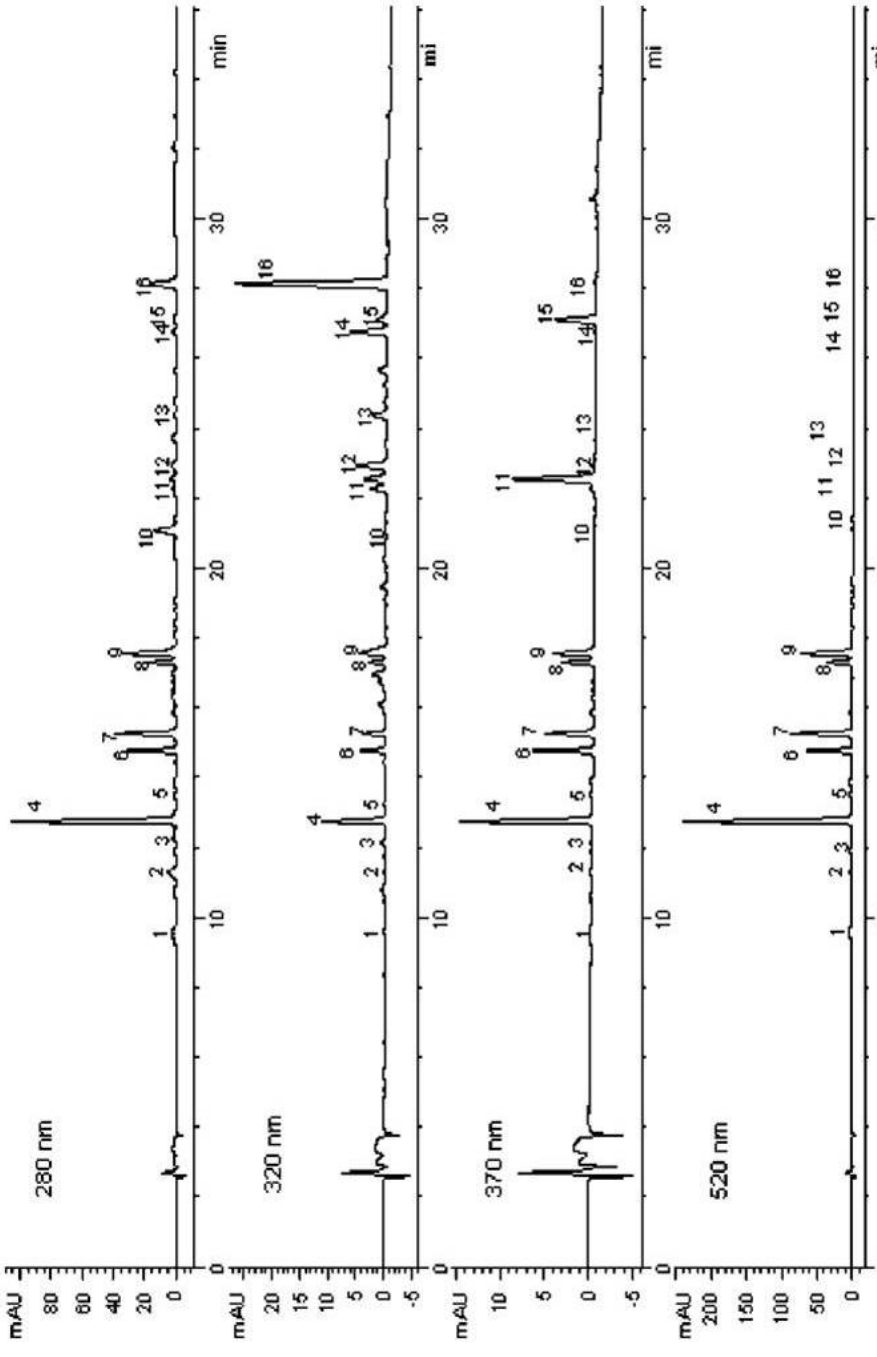


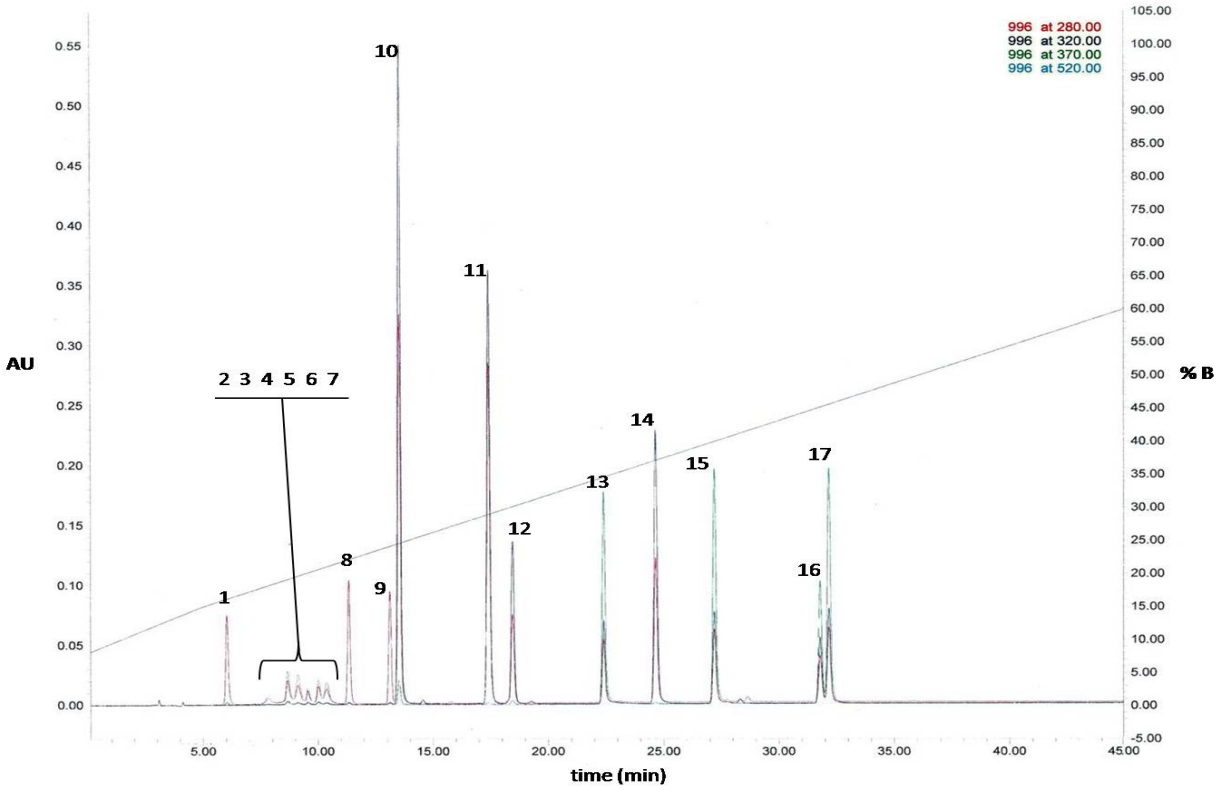
Figure 1. HPLC chromatogram for Noiret whole fruit polyphenolic extract at 280, 320, 370, and 520 nm (different scale size) (mAU = milli absorbance units): 1, procyanidin B1; 2, catechin; 3, procyanidin B2; 4, delphinidin; 5, epicatechin; 6, cyanidin; 7, petunidin; 8, peonidin; 9, malvidin; 10, unknown; 11, myricetin; 12, unknown; 13, resveratrol; 14, unknown; 15, quercetin; 16, unknown.

Table 1
Concentrations of anthocyanins in grape pomace extracts of Sicilian cultivars[a]

Compound[b]	NA	NM	NC	FR	CS
	mg/g[c]				
Delphinidin 3-*O*-glucoside (**4**)	0.21 (0.02)	1.17 (0.15)	0.81 (0.08)	0.21 (0.02)	0.16 (0.01)
Cyanidin 3-*O*-glucoside (**1**)	t	0.64 (0.02)	t	0.09 (0.01)	nd
Petunidin 3-*O*-glucoside (**6**)	0.43 (0.05)	1.43 (0.06)	1.30 (0.16)	0.38 (0.04)	0.32 (0.02)
Peonidin 3-*O*-glucoside (**2**)	0.20 (0.02)	1.48 (0.05)	0.42 (0.06)	0.39 (0.05)	0.15 (0.03)
Malvidin 3-*O*-glucoside (**8**)	5.61 (0.36)	4.09 (0.61)	10.38 (0.51)	2.16 (0.09)	5.70 (0.08)
Delphinidin 3-*O*-(6″-*O*-*p*-coumaryl)-glucoside (**5**)	0.81 (0.11)	nd	2.25 (0.18)	nd	nd
Petunidin 3-*O*-(6″-*O*-*p*-coumaryl)-glucoside (**7**)	1.22 (0.17)	nd	nd	nd	nd
Peonidin 3-*O*-(6″-*O*-*p*-coumaryl)-glucoside (**3**)	0.22 (0.01)	nd	2.97 (0.30)	t	t
Malvidin 3-*O*-(6″-*O*-*p*-coumaryl)-glucoside (**11**)	19.32 (0.02)	0.29 (0.02)	27.14 (2.18)	0.43 (0.01)	0.99 (0.02)
Malvidin 3-*O*-(6″-*O*-acetyl)-glucoside (**9**)	0.68 (0.05)	nd	nd	nd	2.02 (0.16)
Malvidin 3-*O*-(6″-*O*-caffeyl)-glucoside (**10**)	t	nd	nd	0.10 (0.01)	0.27 (0.01)

[a] NA = Nero d'Avola; NM = Nerello Mascalese; NC = Nerello Cappuccio; FR = Frappato ; CS = Cabernet Sauvignon.
[b] Compounds are listed according to the elution order in the HPLC analysis. Standard deviation ($n = 3$) in parentheses; nd = not detected; t = trace (<0.05 mg/g).
[c] Referred to 1 g of MeOH extract.

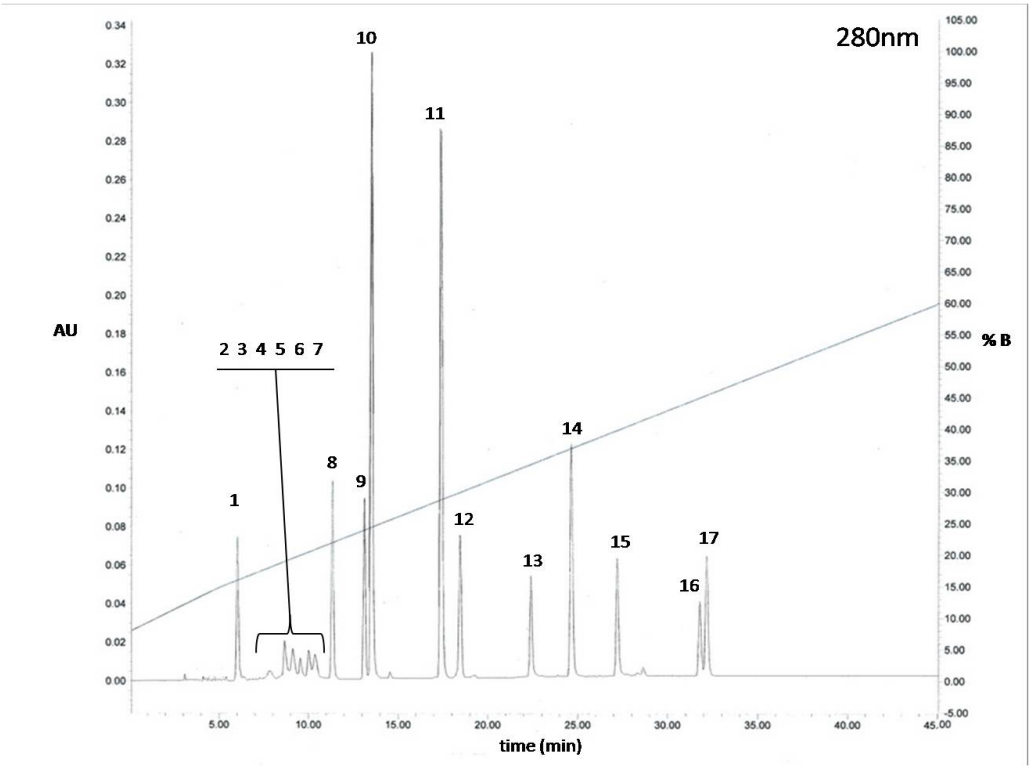
Waters HPLC chromatogram overlay of standards mix at all wavelengths used (280, 320, 370 and 520nm).



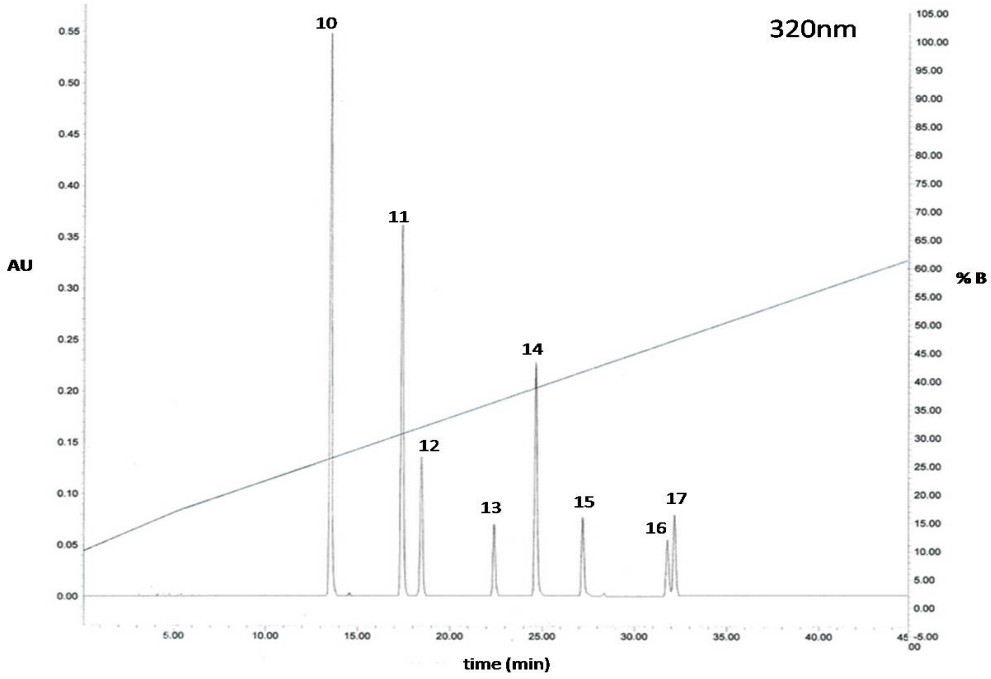
HPLC standards used for identifications of phenolic compounds (Waters system).

	Peak #	Compound	Retention Time (min)	λ used (nm)
Phenolic Acids	1	Gallic acid	6.029	280
	10	Caffeic acid	13.527	320
	11	Coumaric acid	17.406	320
	12	Ferrulic acid	18.461	320
Anthocyanins	2	Delphinidin-3-*O*-glucoside	7.827	520
	3	Cyanidin-3-*O*-glucoside	8.674	520
	4	Petunidin-3-*O*-glucoside	9.131	520
	5	Pelargonidin-3-*O*-glucoside	9.553	520
	6	Peonidin-3-*O*-glucoside	10.018	520
	7	Malvidin-3-*O*-glucoside	10.373	520
Anthoxanthins and Stilbenes	8	Catechin monohydrate	11.337	280
	9	Epicatechin	13.126	280
	13	Myricetin	22.403	370
	14	Resveratrol	24.654	320
	15	Quercetin hydrate	27.205	370
	16	Kaempherol	31.793	370
	17	Isorhamnetin	32.173	370

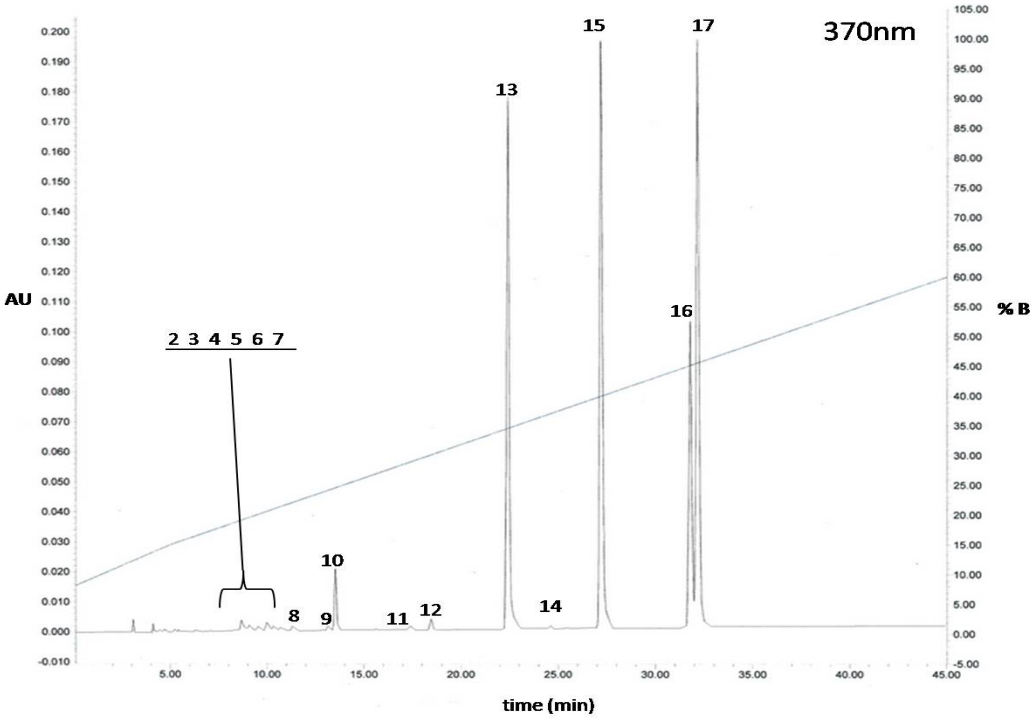
HPLC chromatogram of standards mixture at 280nm (Waters system).



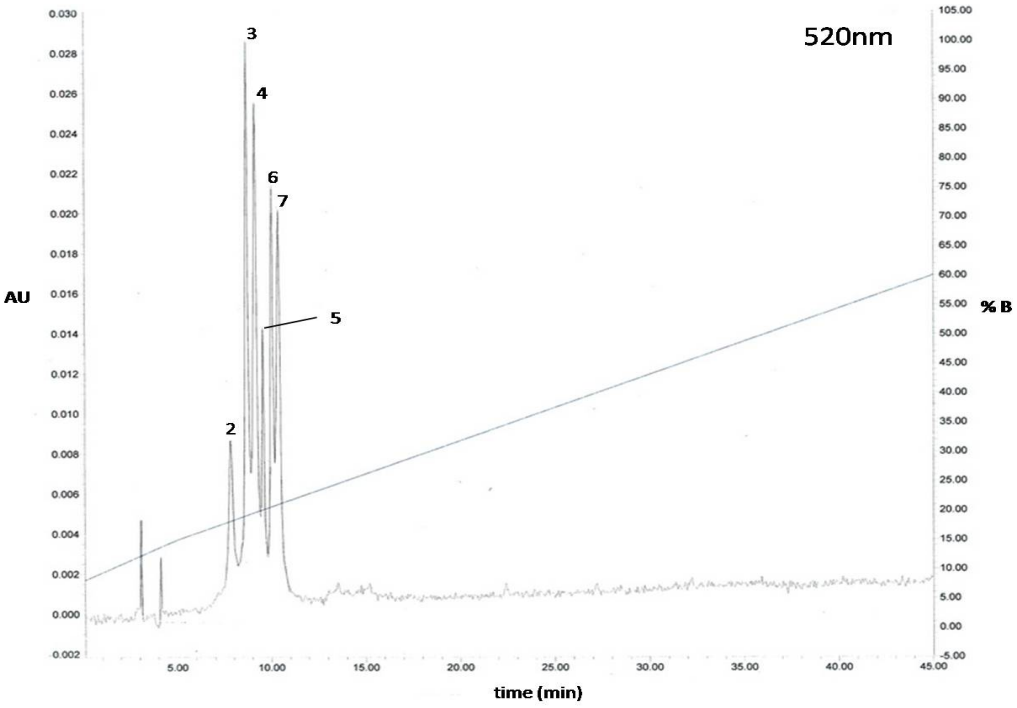
HPLC chromatogram of standards mixture at 320nm (Waters system).



HPLC chromatogram of standards mixture at 370nm (Waters system).



HPLC chromatogram of standards mixture at 520nm (anthocyanins) (Waters system).



VITA

Ioannis Oikonomakos

Candidate for the Degree of

Doctor of Philosophy

Dissertation: INFLUENCE OF SOLVENT EXTRACTION AND WINEMAKING STEPS ON ANTIOXIDANT ACTIVITY OF RED GRAPES

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- American Association of Cereal Chemists (2005)
- American Society for Horticultural Science (2004, 2008)
- Gamma Sigma Delta (ΓΣΔ), the honor society of Agriculture (2007)
- Pi Alpha Xi (ΠΑΧ), Horticulture Honor Society (2006)
- Phi Kappa Phi (ΦΚΦ), National Honor Society (2005

Name: Ioannis Oikonomakos Date of Degree: December, 2009

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Title of Study: INFLUENCE OF SOLVENT EXTRACTION AND WINEMAKING STEPS ON ANTIOXIDANT ACTIVITY OF RED GRAPES

Pages in Study: 87 Candidate for the Degree of Doctor of Philosophy

Major Field: Food Science

Scope and Method of Study: Compare different solvent combinations on antioxidant extraction from Cynthiana pomace for potential industrial scale use. Measure the antioxidant activity of Cabernet franc grapes at various steps during wine production. Identify major compounds present in pomace and wine; correlate antioxidant activity with total phenolics present.

Findings and Conclusions: Out of the 32 solvent treatments, 50% Acetone/Water mix proved to be a good extraction medium when used for 2h at 2:1 solvent:sample ratio. Yield of 31μmoles TE/g tissue suggest that Cynthiana pomace is a good source of natural antioxidants, with anthocyanins cyanidin 3-O-glucoside and malvidin 3-O-glucoside being two of the major compounds in the extracts.

Both wine and pomace of Cabernet franc showed high antioxidant activity of approximately 27μmoles TE/mL wine and 56-83μmoles TE/g pomace respectively. Antioxidant activity was also positively correlated with total phenolic content, both for wine and pomace, with average of 762mg Gallic acid equivalents/L of wine and 2mg Gallic acid equivalents/g pomace respectively. The major compounds identified in wine with HPLC were gallic acid and epicatechin, with malvidin 3-O-glucoside in smaller amounts. Pomace extracts contained mainly catechin and epicatechin, with malvidin 3-O-glucoside as the major anthocyanin. Several major peaks were not identified, which suggests that additional compounds might also be responsible for the antioxidant properties of Cabernet franc.

Both experiments suggest that grape pomace from red grape varieties has good antioxidant properties and thus may be a good source of valuable natural compounds. Our method presents a potentially industrial-scale, single-solvent extraction process for screening antioxidants from grape pomace – and possibly other waste streams. Quantification and identification of all major compounds present in the grape pomace is needed to give a better understanding of the true value of the waste streams and also to help determine if further extract processing would be beneficial and/or economically feasible.

ADVISER'S APPROVAL: Dr. William McGlynn