CHARACTERIZATION OF PHENOLIC COMPOUNDS IN CYNTHIANA GRAPE (*Vitis aestivalis)*

By

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COMPOUNDS IN CYNTHIANA

GRAPE (*Vitis aestivalis)*

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CHAPTER I

INTRODUCTION

Plant phenolics have attracted intense interest from researchers and the public due to their beneficial properties. The research of polyphenols has had focus towards the structural characterization of individual phenolic components in a wide variety of plant matrices and towards the search of sources of phenolic compounds, which might be used for their cost-efficient recovery. Fruits and berries contain a variety of phenolic compounds, which are often located in the external layer of the plant, fruit or berry. The external location of phenolic is associated with their main natural function to protect the plant against environmental stress and pathogens. Plant phenolics include simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, hydrolyzable and condensed anthocyanins , lignans and lignins (Puupponen-Pimiä 2001). Anthocyanins are directly responsible for the color in grapes and wines. Numerous studies have concluded that flavonols and anthocyanins may be used as molecular markers for the classification and differentiation of grape cultivars and single cultivar wines (Castello-Munoz N 2007). Ozimina (1979) also found that either flavonols or the anthocyanins profiles seem to be closely related to the distinctive genetics of the grape. Nevertheless, other studies have evidenced variations in the occurrence of either anthocyanins or flavonols with seasonal

conditions or viticultural practice (Ryan and Revilla 2003).

Phenolic compound extraction is mainly influenced by their chemical nature in plant material, the extraction method, sample particle size, and storage time. Phenolic extracts of plant material have been demonstrated to have a mixture of different classes of phenolics that are soluble in a range of solvent systems. Additional steps may be required for the removal of unwanted phenolics and non-phenolic substances such as waxes, fats, terpenes and chlorophylls. Solid phase extraction (SPE) techniques and fractionation based on acidity are commonly used to remove non-phenolic substances (Tempel 1982).

Reversed phase HPLC using analytical size column (4.0-4.6 mm I.D.) and photodiode array detection has been extensively reported for the identification and quantification of phenolic compounds in grapes and wine. Nevertheless, most of these methods target only a part of classes of phenolic compounds whereas, simultaneous determination of all compounds is fairly unusual (Lin and Harnly 2007). Adsorption technology using non-polar styrene is utilized for purification and fractionation of bioactive compounds in grapes. The phenolic extracts can be partially purified using ion exchange resins, as described by Tibor and Francis (Tibor and Francis 1968). Amberlite particles (XAD-2) have also been utilized for isolation and purification of phenolics present in aqueous plant extracts (Llorach and others 2003). Antimicrobial activity of plant phenolics has been intensively studied, and in addition to controlling invasion and growth of plant pathogens, their activity against human pathogens has been investigated to characterize and develop new healthy food ingredients, medical compounds and pharmaceutical products (Rauha and others 2000). Relatively few data, however, are available concerning the antimicrobial mechanisms from grape derived

purified compounds, although growth inhibitory and antimicrobial activity of grape samples has been reported (Puupponen-Pimia and others 2005; Rauha 2000).

The purpose of this work had as objectives:

- To identify and quantify the major bioactive compounds in the extracts of Cynthiana whole grape, juice, grape pomace and commercial dried grape pomace using High Performance Liquid Chromatography and Mass spectrophotometry (HPLC -MS).
- To identify and quantify the major bioactive compounds in the extracts when Cynthiana whole grape, juice, grape pomace and commercial dried grape pomace is extracted using 50% methanol-water mixture, 50% acetone-water mixture, petroleum ether or 0.01% pectinase solutions.
- To evaluate antibacterial activity of phenolic compounds isolated by Amberlite XAD-7 HP and ion exchange resins in Cynthiana pomace.

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CHAPTER II

LITERATURE REVIEW

Chemical composition of grape

The chemical composition of grapes differs according to variety and the environment in which grapes are grown. Research has demonstrated that environmental factors such as temperature, soil fertility, moisture and light can all have distinct influences on chemical composition (Mullins and Williams, 2003).

Sugars

The main sugars in grapes are glucose and fructose, which play a major role in the fermentation of grapes to produce wine. During wine fermentation, sugars are converted to ethanol and carbon dioxide by yeast (Mullins and Williams, 2003).

Organic acids

There are three primary acids found in wine grapes: tartaric, malic and citric. Throughout the process of winemaking and in the final wines, butyric, acetic, lactic and succinic acid can play significant roles, such as maintaining the growth and vitality of yeasts during fermentation and protecting wine from bacteria (Mullins and Williams, 2003).

Aroma compounds

Many volatile odorous compounds are found in wine. These aromatic substances are derived from three major sources:

1. Grapes (fruits)
2. Fermentation
3. Aging and maturation

Grapes contain numerous flavor compounds. Some of these compounds have been reported to give a variety their distinct varietal character. Examples of these include: a. 2-methoxy-3-isobutyl pyrazine - Predominant compound giving bell pepper-like odors to varieties like Cabernet Sauvignon and Sauvignon blanc. b. 4-vinylguaiacol and 4-vinylphenol - giving spicy, clove-like and medicinal odors (Castello-Munoz 2007).

Nitrogenous compounds

Grapes contain various nitrogenous compounds. These include ammonium cations and organic nitrogenous compounds: such as amino acids, peptides, and proteins. The nitrogen content of the grape varies with variety, climate, soil, fertilization, and other cultural practices. The total nitrogen concentration of the fruit increases during the maturation period (Keller 1998).

Nitrogen containing compounds are important because they serve as the nutrient for yeast and lactic acid bacteria. Nitrogen influences biomass formation (cell population or cell yield), rate of fermentation, and production of various byproducts, which in turn affects the sensory attributes of wine. Proteins (nitrogenous compounds) are involved in wine stability. Insufficient nitrogen in must can cause a sluggish or stuck fermentation and the formation of a "rotten egg" (H_2S) odor. To avoid such a problem, the must is often supplemented with diammonium

phosphate (DAP). The maximum amount of DAP addition legally allowed is 8 lbs/1000 gal or 958.7 mg/L (Keller 1998).

Minerals

Minerals are taken up by the vine from the soil. They usually make up approximately from 0.2 to 0.6% of the fresh weight of the fruit. The important mineral compounds include: potassium, sodium, iron, phosphates, sulfate, and chloride. Of the mineral compounds mentioned above, potassium is the most important mineral. It accounts for 50 to 70% of the cations in the juice. During ripening, the potassium content of the grape increases. Its movement into fruit leads to the formation of potassium bitartrate, which reduces the acidity and increases the juice pH. It should be noted that the tartaric acid salt of potassium is involved in wine instability problems (Harborne 1988).

Pectic Substances

Pectin substances are cementing agents present in the cell wall. Chemically, they are complex polysaccharides made of galacturonic acid molecules linked together. During ripening, pectin is hydrolyzed by naturally occurring pectolytic enzymes, which renders the berry softer as it ripens. In juice, the pectin causes cloudiness by holding the particles of fruit pulp in suspension. To allow the suspended solids to settle and clarify the juice, commercial preparations of pectolytic enzymes are often used (Ryan and Revilla 2003).

Phenolic compounds

Phenolic compounds are defined as a group of chemical compounds consisting of a hydroxyl functional group (-OH) attached to an aromatic hydrocarbon group (Strack 1997). The simplest one is phenol (C_6H_5OH). Based on their structures two groups namely flavonoids and non-flavonoid phenols are distinguished (Figure 1) (Bowyer 2002b). Flavonoid phenols are subdivided into anthocyanins, flavanols, flavonols and tannins (Kennedy 2001; Allen 1998).

Non-flavonoid phenols consist primarily of phenolic acids and their esters (Singleton 1976). The differences between compounds from both groups are the number and orientation of phenolic sub-units within the molecules.

Phenolic compounds in red grape and wine

Phenolics are extensively distributed in plants and they have been studied for the several decades for their antioxidant and antimicrobial properties (Harborne 1988). These compounds have an broad diversity: more than 4,000 compounds have been identified and they are present in the environment as a result of their uses and the process in which they are implicated (Strube 1993). Phenolics have been linked to several functions in plants: protection from UV radiation, pigmentation, defense against invading pathogens, nodule protection, and attraction of pollinators and seed dispersers (Lu 1997) . Plants generate a great variety of natural compounds that are not directly involved in primary metabolic processes of growth and development. The roles of these secondary metabolites in plants have recently come to be valued in an analytical context.

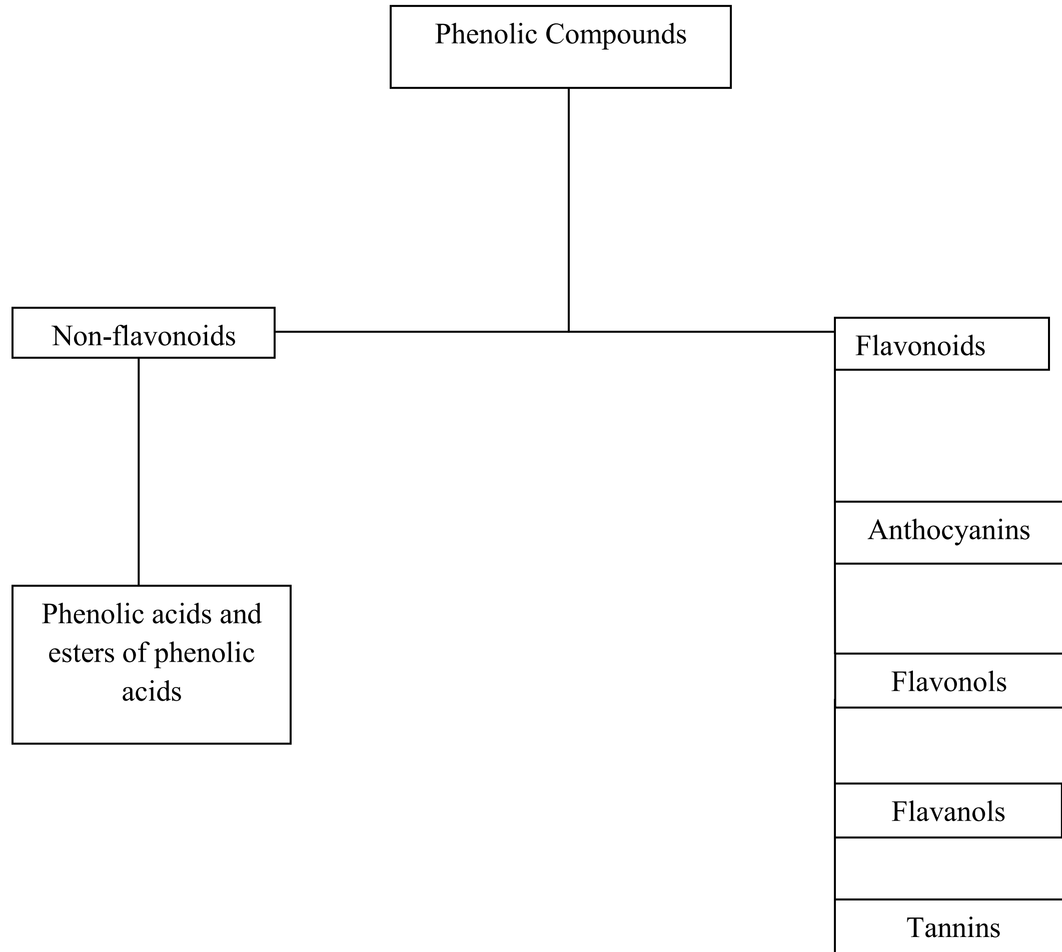


Figure 1. Classification of phenolic compounds based on their structure

Non-flavonoids

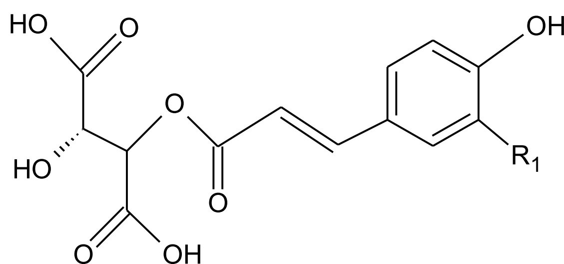
Phenolic acids

Benzoic and cinnamic acids are phenolic acids present in grape skins and pulp (Riberean-Gayon 1965). Although concentrations of most non-flavonoids are below their individual sensory thresholds values, collectively some of these compounds may contribute to the sensory sensations of bitterness and harshness (Gawel 1998; Nagel 1981)

.

Phenolic acids are divided in the basic of their carbohydrate backbone in hydroxycinnamic acids (C_3-C_6) or hydroxybenzoic acids (C_1-C_6). The most common phenolics acids found in grapes are shown in Figure 2 (Macheix 1990a).

Hydroxycinnamic acids	R_1	R_2	Hydroxybenzoic acids	R_1	R_2
p-coumaric acid	H	H	Gallic acid	OH	OH
Ferulic acid	O	CH_3	Protocateic acid	OH	H
Sinapic acid	OCH_3	OCH_3	Syringic acid	OCH_3	OCH_3
Caffeic acid	H	OH	Vanillic acid	OCH_3	OCH



Hydroxycinnamic acid esters	R_1
Caftaric acid	OH
Coutaric	H
Trans-fertaric acid	OCH_3

Figure 2. Main phenolic acids identified in red grapes.

Stilbenes

Stilbenes are non-flavonoid compounds located in grape berry skins (Jeandet 1991; Lamula-Raventos 1995). A particular stilbene, resveratrol, has attracted general attention due to its healthful properties (Figure 3). Resveratrol compounds have anticarcinogenic and antitumor properties, which reduce the risk of cardiovascular diseases and some types of cancer (Jang 1997; Falchetti 2001). Resveratrol is extracted during fermentation and reported concentrations in wine range from 1 to 50 mg/L (Lamula-Raventos 1995; Ribero de Lima 1999; Baptista 2001; Netzel 2003).

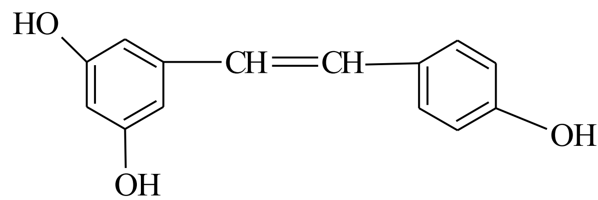


Figure 3. Resveratrol

Flavonoids

Flavonoids are usually categorized as phenolics or polyphenols due to their chemical structure Figure 5. The chemistry of this group is C_{15} (C_6-C_3-C_6) flavones nucleus, two benzene rings linked through an oxygen containing pyran or pyrone ring (Delgado-Vargas 2000).

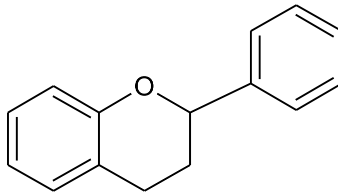


Figure 4. Basic monomeric structure of flavonoids.

Anthocyanins

Interest in anthocyanins has increased because of their potential use as natural colorants and potential beneficial health effects. Regular consumption of anthocyanins and other polyphenols in fruits, wines and jams reduce risks of chronic diseases such as cancer, cardiovascular disease, virus inhibition and Alzheimer´s disease (Middleton, 2000; Bohm 1998; Murkovic 2002; Rice Evans & Parker, 2003). Other factors such as pH, solvents, temperature anthocyanin concentration, oxygen, light, enzymes and other accompanying substances may also influence anthocyanin stability.

Anthocyanin stability depends on the substitution pattern of the aglycone. Glucosyl units and acyl groups attached to the aglycone have a significant effect on their stability and reactivity as well as the number and placement of hydroxyl and methoxyl groups that confer more or lesser stability to the molecule. According to Mazza and Brouillard (1987a; 1987b) a higher number of methoxyl and hydroxyl groups weaken anthocyanin stability. Indeed, methoxyl groups are more

molecule destabilizing than hydroxyl ones. This substitution pattern also influences the colour of anthocyanins. The colour of anthocyanins changes from pink to blue as the number of hydroxyls increase. Glucosilated forms of anthocyanidins are more stable and with a higher rate of occurrence in nature than aglycones (Timberlake & Bridle, 1966a; 1966b).

Also different patterns of hydroxylation and glycosylation appear to modulate anthocyanin antioxidant properties. Hydroxyl groups situated at C3′ and C4′ clearly increase the antioxidant capacity of the anthocyanins. In addition, the glucoside group joined to the flavilium ring affects their antioxidant properties; 3-glucosylation in the C ring increased the oxygen radical absorbance capacity (ORAC) for glucose and rhamnoglucose whereas ORAC values decreased for galactose (Wang 1997). Acylation further increases the stability of anthocyanins (Bassa & Francis, 1987). Polyacylated and aromatic acyl substituents are more stable than monoacylated and aliphatic acyls (Asen, 1976; Francis, 1989).

Anthocyanins are glycosides that release the anthocyanidin aglycone by hydrolysis. The aglycones exist in various forms in pH dependent equilibrium, which impacts their solubility and extraction behavior. Six anthocyanins are commonly contribute to the pigmentation of fruits (Gao 1995a).

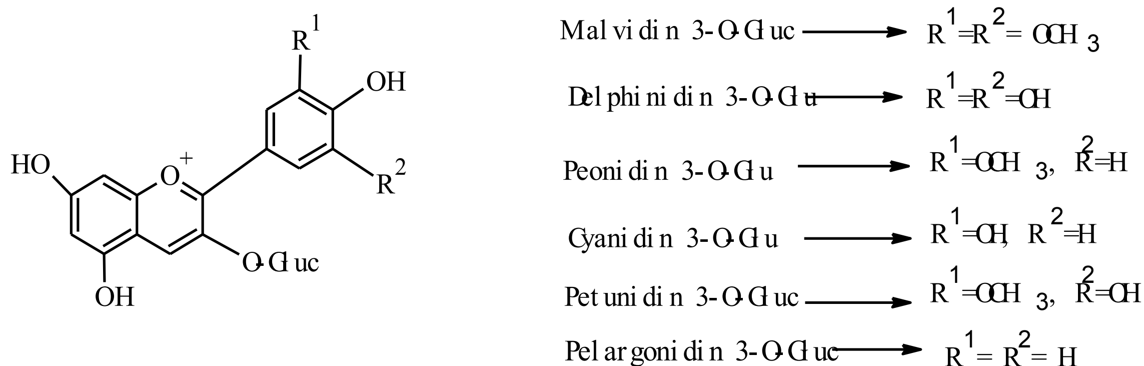


Figure 5. Structure of main 3-O-lgucoside anthocyanins found in grape

Cyanidin is the most common and, in terms of frequency of occurrence, is followed in decreasing order by delphinidin, peonidin, pelargonidin, petunidin, and malvidin. Anthocyanins are traditionally recovered as the flavylium cation by extraction with cold methanol containing hydrochloric acid (Da Costa 2000).

Flavanols

Cathecins or flavan-3-ols are found in the seeds and are known for being bitter (Harbone 1994). Flavanols bitterness in wines is primarily due to the flavan-3-ols, catechin (Figure 6) and its epimer, epicatechin.

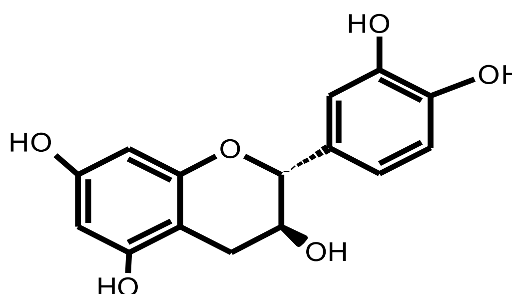
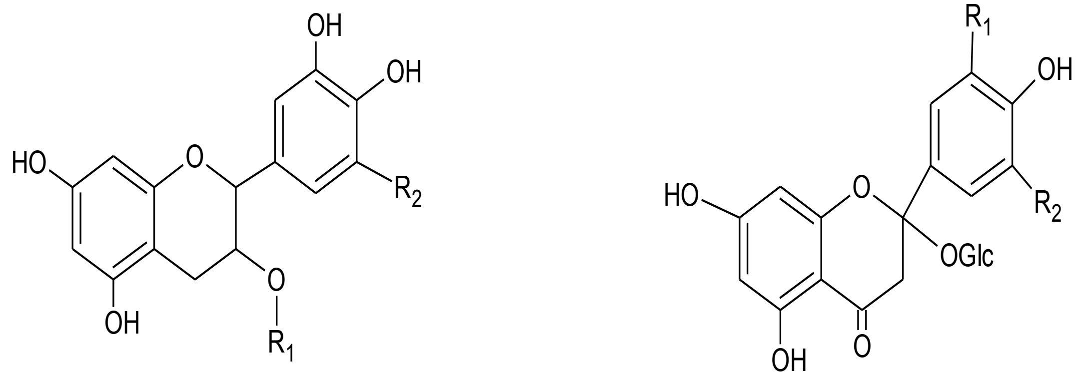


Figure 6. Catechin

Flavonols

The major flavonols in grapes are the 3-glucoside forms derivaed from quercetin, kaempferol, isorhammetin and myricetin mainly found in grape skins (Lu 1997). Other flavonols include different glucoside derivatives.



Flavan-3-ols	R_1	R_2	Flavanols	R_1	R_2
(+)Catechin	H	H	Kaempferol	H	H
(+)Gallocatechin	OH	H	Quercetin	OH	H
(-)Epicatechin	H	H	Myricetin	OH	OH

Figure 7. Common flavan-3-ols and flavonols in grapes

Tannins

Studies have shown the existence of tannin aggregates in the vacuoles of grape skin cells. Most epidermal cells but only few hypodermal cells, which are more abundant in the external layers, contain tannins. Tannins have been mainly localized in vacuole membranes and cell walls (Conn 2003). Anthocyanins and flavonols are mainly localized in skins, except in a few varieties, that also are contained in the pulp. In the skin, they are present in the first external layers of the hypodermal tissue, and exclusively in the vacuoles (Figure 8).

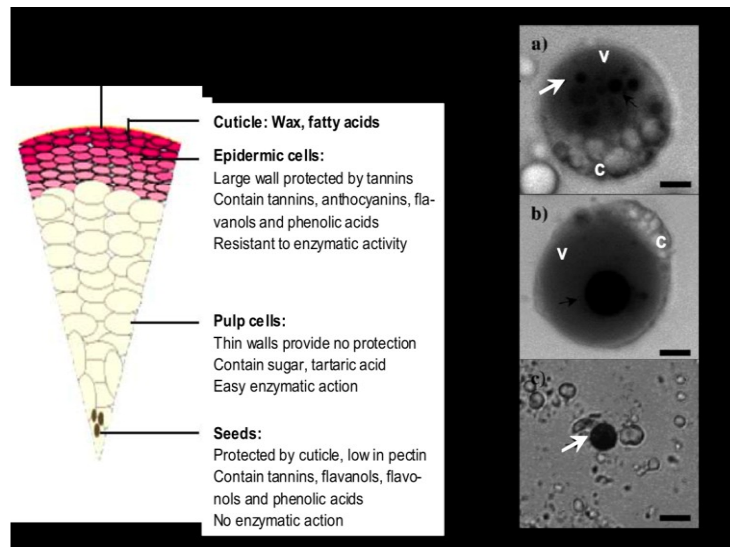


Figure 8. Phenolic compounds location in grapes (Left). Microscopic images of dark-grown V. vinifera L. protoplasts under bright field microscopy (a, b) before and (c) after lysis. Arrows indicate anthocyanin vacuolar inclusion; Bar: 10µm, V: vacuole; C: cytoplasm (Right) (adapted from (Conn 2003).

Biosynthesis of phenolic compounds in plants

The biosynthetic pathways of phenolic compounds in plants have been extensively researched (Haddock 1982) and biosynthesis pathways of some flavonols and phenolic acids are represented in Figure 2. The synthesis of phenolics in plants can be endogenously controlled during plant development and differentiation (Macheix 1990a; Haddock 1982; Harborne 1988; Strube 1993) or it can be regulated by exogenous factors: biotic such as insects and diseases and abiotic, such as light, temperature and nutrient stress (Bennet 1994). The phenylalanine/hydroxycinnamate pathway is defined as "general phenylpropanoid metabolism" and involves the transformation of L-phenylalanine to the hydroxycinnamates and their active forms (Strack 1997) catalyzed by phenylalanine ammonia-lyase (PAL).

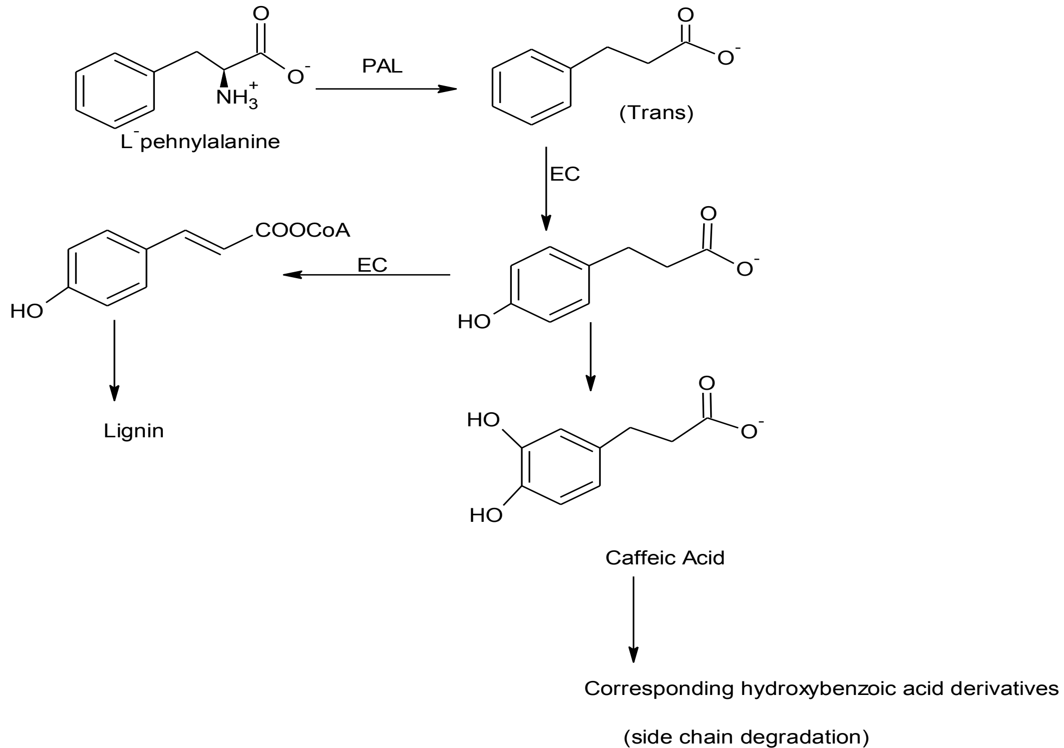


Figure 9. Biosynthetic patways of hydroxycinnamic and hydroxybenzoic acids from L-phenilalanine (Robbins 2003). L-phenylalanine ammonia lyase (PAL), enzymatic conversion (EC)

Red grape wine pomace

Grape growing plays a major role in the worldwide fruit production, with an international acreage of approximately 7.8 million hectares (OIV 2002), and is constantly growing.

Wine industry wastes account for almost 30% (w:w) of the grapes used for wine production (Makris 2007). Grape pomace, a by-product of the wine making process, is

one of the most important residues of the wine industry. It consists of different amounts of grape, skin, pulp, seeds, and, if not removed, stems (Baumgartel 2005). During the vinification process of red wine, phenolic compounds are transferred from solid parts of the grape cluster into wine. The rate of transfer depends on various factors including phenolic concentration of grapes, level of pressing, maceration time, fermentation contact time, temperature, and alcohol levels. The seeds contain the highest concentrations of phenolic compounds and most of these compounds are monomeric flavan-3-ols (catechins) and procyanidins (Sun and others 1999). Both grape skins and seeds contain monomeric, oligomeric, and polymeric proanthocyanidins; the mean degree of polymerization being higher for skin flavanols (Torres and Bobet 2001).

The phenolic compounds of wine, and particularly the flavanols (i.e. catechins), have been the center of attention in recent studies since their relation to the beneficial effects of a moderate consumption of wine was observed (Gonzalez-Paramas 2004). Phenolic compounds present in red wine cause an increase in serum total antioxidant capacity when ingested which thereby inhibit low-density lipoprotein (Yildirim 2005), and

reduce the risk of cardiovascular disease. The antioxidative properties may also exert a chemopreventive role toward degenerative diseases (Ruberto 2005), as well as act as preventative agents against skin cancer and other diseases (Torres 2002).

Extraction of grape pomace phenolic compounds

Grape pomace is usually extracted with organic solvents, most commonly aqueous methanol, ethanol, or acetone.

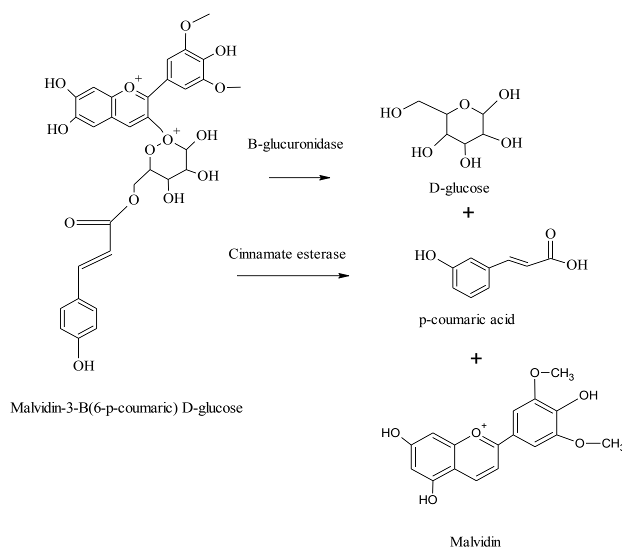
For extraction, Soxhlet extraction is one of the most popular techniques for isolating phenolic compounds from solid samples. This is probably due to its simplicity, inexpensive extraction apparatus and its use in the EPA official methods (Morales and Cela 2000). Polar solvents, such methanol, acetone, acetonitrile to yield high extraction efficiencies. However, they also extract other undesirable polar compounds present in samples (Alonso and others 1998). With apolar solvents, such as n-hexane or dichloromethane, the extraction of phenol requires a previous acidic digestion of the analytes. Satisfactory recoveries of phenols from soils and sediments have been reported with this technique using, in most cases, mixtures of polar and apolar organic solvents (Rodríguez and others 2000). Alonso (Alonso et al. 1998) developed an analytical protocol for the determination of priority phenolic compounds in soil samples using a solvent mixture, methanol-water (4:1), both containing 2% triethylamine, to enhance the extraction of more chlorinated phenols. Recoveries varied in the range from 67-97% with a standard deviation between 8 and 14%. Despite, the results obtained with this methodology, Soxhlet extraction makes the analysis procedure excessive time consuming. Moreover, it requires large amounts of hazardous organic solvents.

Hydrolysis

Hydrolysis was described by Markham as an aid to identify and quantify glycosides in fruits (Markham 1982). The use of hydrolysis has been common as an aid to simplify chromatographic data, principally in instances where appropriate standards are commercially unavailable and also to minimize interferences in subsequent chromatography. Several forms of hydrolysis have been used for this purpose: acidic, enzymatic and alkaline. Chemical treatment has been more common because it is less selective and more destructive (Abou-zaid and Nozzolillo 1991). Hydrolysis methods are used for purposes other than categorization/structural explanation of unknown phenols result in a reduction in information content. A sample extract containing several *O*-glucosides of a single aglycone plus the free aglycone will produce after acid hydrolysis a single HPLC peak. The advantages in terms of simplicity of interpretation and quantification are apparent as seen in HPLC of grape pomace (Rommel 1993) where acid and base hydrolysis simplified the complex phenolic profiles dramatically. There is a considerable variation of the glycosidic bond under hydrolytic conditions. The rate of acid/base hydrolysis of glycosides depends on acid/base strength, the nature of the sugar and the position of attachment to the flavonoid nucleus. On the other hand, enzymatic hydrolysis is made from commercial pectolytic enzymes which are commonly prepared from the extracellular material of *Aspergillus* or *Trichoderma* species. These enzymes preparations are rather crude, containing several pectinase activates as well as containing several side activities that may attack both the phenolic glycoside bonds and other bonds in the plant material. The variable and contradictory results of pectolytic preparation used for color extraction and stability in red wines, have been attributed to side activities in the enzyme preparation, most notably is the side activity of β-glucuronidase. This activity was first identified in *Aspergillus* sp. enzyme extracts by Huang (1955), who found

it to cause discoloring activity to anthocyanin in different fruits. Wrolstad (1994) screened some of the commercial red grape processing enzymes for this destructive β-glucuronidase activity. They identified some enzymes that appear to have this side activity, and some enzymes that do not have any side activity. β-glucuronidase cleaves the glucose from position three of the anthocyanin producing anthocyanidin and glucose (Figure 9), followed by the spontaneous transformation of unstable aglycone to the colorless chalcone form, then degradation products. The mechanism of the degradation of an anthocyanin by the β-glucuronidase enzyme is shown in Figure 10.

Figure 10. Enzymatic hydrolysis of a typical grape anthocyanin. Modified from Zoecklein 1990



Quantification procedures

Spectrophotometric methods

The total phenolic content of grape pomace is generally determined colorimetrically with a UV-vis spectrophotometer using Folin-Denis (Singleton 1965). The main principle of these assays is similar based on the reduction of phosphomolybdic-phosphotungstic acid reagent (folin reagent) in alkaline solution (Singleton 1965). During the assay, the methanolic and water-based

solutions of grape pomace samples, folic reagent and sodium carbonate solution are mixed and after 30 min the absorbance is measured at 725-765 nm (Singleton 1965).

Thin layer chromatography

Thin layer chromatography (TLC) has been applied in separation and identifying the phenolic acids in different plants (Ryan and others 2002). Usually TLC is used to control the purity of samples by gas chromatography. The TLC plates are coated with silica gel and fluorescent indicator UV-254 (Akillioglu 1997). This technique has also been used to separate individual phenolic compound such as glucopyranosyl sinapate and other phenolic compounds from grape pomace (Rapp 1973). Quantification of the compounds requires some additional methods, such as purification with amberlite resins and semipreparative HPLC.

Gas chromatography

Many studies have been used for identification and quantification where gas chromatography plays an important role. In most cases chromatograph has been equipped with a flame ionization detector and packed glass column with 1.5 % SE-30 and nitrogen as a carrier gas (Markham 1982). The chromatographic run has usually been temperature programmed, e.g. from 120 to 300°C at 4°C/min, 130-12°C at 5°C/min.

High performance liquid chromatography

High performance liquid chromatography (HPLC) has become a common replacement for gas chromatography. The HPLC system has usually been prepared with UV detector and a reverse phase column C_{18} column. In contrast, reversed-phase HPLC avoids the need for derivatization and has invariably been the method of choice. Isocratic elution has been used in

some instances (Da Costa 2000) but the procedures regularly rely on gradient elution remaining to the diversity of phenols in most extract. Typical mobile phases include methanol, water, acetonitrile and acetic acid combinations that are used in gradient elution techniques (Lamuela-Raventós 1994; Montedoro 1992). Detection of bioactive compounds from plants by HPLC is based on measurement of adsorption of radiation in the UV or visible region. The most common wavelengths for general detection has been 280 nm (Tsimidou and others 1992) although other wavelengths have been used for the identification of specific phenolic (Esti 1998; Daigle 1988). For example, phenols have been quantified at characteristic wavelengths as cinnamic acids (320 nm), flavonols (360 nm) and other phenols (280 nm) (Suárez and others 2005). The paucity of reference compounds creates difficulties in quantification that have been solved by normalization (Rommel 1993), synthesis or isolation from the sample (Romani 1999) of the relevant phenols or the use of phenol belonging to the same class (Keller 1998).

Capillary zone electrophoresis

In capillary zone electrophoresis (CZE), the buffer composition is constant through out the region of the separation. The applied field causes each of the different ionic components of the mixture to migrate according to its own mobility and to separate into zones that may be completely resolved or that may partially overlap. Completely resolved zones have region or buffers between them. The situation is analogous to elution column chromatography, where regions of mobile phase are located between zones containing separated analytes. Other methods have been reported (Ayaz and others 1997) but have not found general acceptance. For instance, capillary electrophoresis (CZE) and micellar electrokinetic capillary chromatography have been used to separate phenolic compounds (Da Costa 2000). The majority of these separations used buffers at pH 8.0-10.5 that are suitable for the majority of phenols with pKa values between 8

and 10 but are unsuitable for pH-sensitive anthocyanins. Anthocyanins were measured in blackcurrant juice (Da Costa 2000) by CZE under strongly acidic conditions favoring the red-coloured flavylium cationic form. Under these conditions, the Anthocyanins absorb at 520 nm.

Ion exchange chromatography

Ion exchange chromatography is composed of a polymer matrix, based on inorganic compounds and a functional group. Depending on the positive or negative charge of the ion-active group, the ion is separated as cation or anion and depending on the affinity for counterions each type can be act as strong or weak exchangers. The strong cation exchangers contain sulfonic acid functional groups, and the weak cation exchange column packing carboxylic acid functional groups. The strong anion exchange has quaternary ammonium group and the weak anion exchangers have tertiary amine substitutes. Pure phenols and natural extracts were absorbed onto strong and weak anion and cation exchange resins (Ku 2004; Ramam 2005; Carmona 2006). Introducing suitable functional groups onto a resin may result in improved selectivity and efficiency (Yan and Tan 2008; Geng 2009;). Molecular imprinted polymers are also applicable, combining high selectivity and affinity for the adsorbates, low cost, physical resitance and durability (Ersoz 2004; Lin 2008).

Biological Properties of Grapes and Wine

Although, phytochemistry of *Vitis vinifera* is very attractive to scientists, there is also a great interest for the beneficial effects of that plant and its metabolites (Renaud 1992; Torres 2002; Murthy 2002).

Antioxidant activity

It is known that grapes are rich in antioxidants substances such as flavonoids, stilbenes, anthocyanins, etc. Extracts mainly from red grapes have demonstrated significant antioxidant activity which is growing in proportion to the containing percentage of polyphenolic molecules (Negro 2003). In addition extracts from seeds, bark and from whole grapes have great obstructive activity against free radicals (Caillet 2006). Another category of substances, found in grapes, are the stilbenes. In particular, resveratrol, the main representative of stilbenes, has strong antioxidant activity and protects body from oxidative stress (Li Y 2006). The same activity against free radicals is also shown by anthocyanins which are responsible for the coloration of grapes (Stintzing & Carle 2004).

Anthocyanins, in synergy with other antioxidants of grape extracts, by preventing the oxidation of cells in pancreas, which produces insulin, becomes a protective agent against occurrence of diabetes (Rahimi 2005).

Protective effect of cardiovascular diseases

Beneficial effect of wine is a phenomenon that appeared first in the international literature in 1992 (Renaud S. & De Lorgeril 1992) and called “French Paradox”. The principle for the formulation of this phenomenon was the conduct of worldwide clinical research on the relationship between a diet rich in fat and many calories with mortality from coronary heart disease. This relationship was found to be similar. However, in some regions of France, the mortality was markedly lower (about 1/3) than in the U.S. and Great Britain and was closer to statistics in China and Japan. This initially seemed paradoxical, since the daily intake of fat from the French was the same as in the other countries. Therefore, scientists have sought to distinguish the difference in the diets of these countries. After more extensive studies it was found that the

French have lower rate of heart disease, because of the larger amount of red wine consumption at meals.

It has been reported the protective role of grape extract in situations of ischemic attack. (Gross 2005). Both in vivo experiments and clinical trials have demonstrated the ability to scavenge free radicals, the protection of myocardial cells, the reduction of athirogenesis possibility and generally better heart function (Bagchi 2003). Polyphenols that have been isolated from grapes, have a beneficial effect on hypertension, coronary heart disease and other cardiovascular diseases. It was found an inverse relationship between intake of polyphenols and the occurrence of cardiovascular diseases (Dell' Agli 2004; Stoclet 2004). In addition flavonoids reduce platelet aggregation and prevents oxidation of low-density lipoproteins (LDL), resulting in the reduction of blood cholesterol levels and reducing the risk of cardiovascular diseases (Frankel 1995; Teissedre 1996). Such activity shows also and Resveratrol (Ethernon 2002; Mayer 1997).

Anticancer activity

Vitis vinifera chemoprotective activity has been tested in *in vivo* experiments on skin cancer of mice in which tumours were created on skin surface. It proved that ethanol grape extract prolong the latency of tumours and reduces their number (Alam 2002). Besides, many food components which are beneficial against cancer, one found in grapes (resveratrol, quercetin etc.) (Chen & Kong 2005).

Several *in vivo* and *in vitro* studies have shown that grape extracts showed cytotoxicity towards cultured human cancer cells (e.g. breast and lung cancer cells) as well as inhibited human prostate tumor xenograft growth in mice (Agarwal 2000; Singh 2004). Flavonoids of grapes can also affect the metabolism of foods cancer-causing agents and inactivate them

(Stavric 1994). Resveratrol also shows significant anticancer activity and is considered one of the most effective antineoplastic molecules of plant origin. It can be applied in regimens with traditional cancer drugs, in order to increase the sensitivity of tumour to them and reduce their side effects (D' Incalci 2005).

Antimicrobial activity

Grape extracts have shown antibacterial action in several *in vitro* experiments, which is attributed to containing polyphenolic metabolites. In particular, red grape extracts were tested against 15 strains of microorganism such as *Bacillus Brevis, Escherichia coli, Pseudomonas aeruginosa* etc. Results were very encouraging since it was demonstrated that the extracts were active at concentrations of 4% and 20%. This action is particularly important as the grape extracts may be used as antibacterial agents to protect food from spoilage caused by bacteria. Grape seeds and pomace extract have also been studied for their antimicrobial activity (Jayaprakasha 2003; Baydar 2004). Recently Anastasiadi (Anastasiadi 2009) reported the antilisterial acivity of grape berries extracts rich in polyphenols and vification byproducts, obtained from Greek islands *Vitis vinifera* varietes.

Purification and isolation of phenolic compounds

Amberlite XAD

Adsorption applications using Amberlite polymeric adsorbents may be carried out using either column or batch techniques. Column operations are generally preferred although the choice of method depends entirely upon the application in question (Tomás-Barberán 1992).

When treating most solutions, the polymeric adsorbent is used in a vertical column to from an adsorption bed. The solution to be treated is allowed to flow through the column until a target end point is reached. At this point, the adsorbent is regenerated to prepare it for another cycle. An adsorption column may be viewed as a series of equilibrium stages, much like the

theoretical plate theory in distillation. As solution proceeds down the column, the adsorption reaction is continuous driven in the forward direction by equilibrium requirements of each new theoretical stage encountered (Puupponen-Pimia 2005).

Polymeric adsorbents are highly porous structures whose internal surfaces can adsorb and then desorb a wide variety of different species depending on the environment in which they are used. For example, in polar solvents such as water, polymeric adsorbents exhibit non-polar or hydrophobic behavior and so can adsorb organic species that are sparingly soluble. This hydrophobicity is most pronounced with the styrene adsorbents. In non-polar solvents, such as hydrocarbons, etc. most adsorbents exhibit slightly polar or hydrophilic properties and so will adsorb species with some degree of polarity. This polarity is most pronounced with the acrylic adsorbents and the phenolic adsorbents (Bennet 1994; Harbone 1994; Morales and Cela 2000; Ryan 2002).

Purification strategies are developed to utilize these performance characteristics. In capture/concentration mode, Amberlite™ XAD™ media provide an excellent first purification step in the recovery of antibiotics from complex fermentation broths. In the same application XAD™ resins perform decolorization and desalting functions as well. The relative large pores of XAD make it an ideal candidate for the adsorption of large molecules from plant extracts or other natural sources (Tomás-Barberán 1992). Elution can be performed either with solvents, buffers or steam depending on the type of molecules under consideration.

Ion exchange

Ion exchange (IEX) is one of the most frequently used techniques for purification of proteins, peptides, and nucleic acids and other charged biomolecules, offering high resolution and group separations with high loading capacity. The technique is capable of separating molecular species that have only minor differences in their charge properties, for example two

proteins differing by one charged amino acid (Ku 2004; Ramam 2005; Carmona 2006). These features make IEX well suited for capture, intermediate purification or polishing steps in a purification protocol and the technique is used from microscale purification and analysis through to purification of kilograms of product. IEX separates molecules on the basis of differences in their net surface charge (Ersoz 2004; Lin 2008). Molecules vary considerably in their charge properties and will exhibit different degrees of interaction with charged chromatography media according to differences in their overall charge, charge density and surface charge distribution. The charged groups within a molecule that contribute to the net surface charge possess different *pKa* values depending on their structure and chemical microenvironment.

Since all molecules with ionizable groups can be titrated, their net surface charge is highly pH dependent. In the case of proteins, which are built up of many different amino acids containing weak acidic and basic groups, their net surface charge will change gradually as the pH of the environment changes i.e. proteins are *amphoteric*. Each protein has its own unique *net charge versus pH relationship* which can be visualized as a *titration curve*. This curve reflects how the overall net charge of the protein changes according to the pH of the surroundings (Yan and Tan, 2008; Geng, 2009).

An IEX medium comprises a *matrix* of spherical particles substituted with ionic groups that are negatively (cationic) or positively (anionic) charged. The matrix is usually porous to give a high internal surface area. The medium is packed into a column to form a *packed bed*. The bed is then equilibrated with buffer which fills the pores of the matrix and the space in between the particles.

The pH and ionic strength of the equilibration buffer are selected to ensure that, when sample is loaded, proteins of interest bind to the medium and as many impurities as possible do

not bind. The proteins which bind are effectively concentrated onto the column while proteins that do not have the correct surface charge pass through the column at the same speed as the flow of buffer, eluting during or just after sample application, depending on the total volume of sample being loaded (Yan and Tan, 2008; Geng, 2009).

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CHAPTER III

ANALYSIS OF PHENOLIC COMPOUNDS FROM CYNTHIANA GRAPE (*VITIS AESTIVALIS*) BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Abstract

Phenolic acids, flavanols, flavonols and stilbenes (PAFFS) were isolated from Cynthiana (*Vitis aestivalis*) whole grapes, juice, or pomace and purified using enzymatic hydrolysis. Flavonoid-anthocyanins (FA) were isolated using methanol/0.1% hydrochloric acid extraction. In addition, crude extractions of phenolic compounds from Cynthiana grape using 50% methanol, 70% methanol, 50% acetone, 0.01% pectinase, or petroleum ether were also evaluated. Reverse phase high performance liquid chromatography (RP-HPLC) with diode array detector was used to identify phenolic compounds. A method was developed for simultaneous separation, identification and quantification of both PAFFS and FA. Quantification was performed by the internal standard method using a five point regression graph of the UV-visible absorption data collected at the wavelength of maximum absorbance for each analyte. From whole grape samples nine phenolic compounds were tentatively identified and quantified. The individual phenolic compounds content varied from 3 to 875 mg kg^{-1} dry weight. For juice, twelve

phenolic compounds were identified and quantified. The content varied from 0.07 to 910 mg kg^{-1} dry weight. For pomace, a total of fifteen phenolic compounds were tentatively identified and quantified. The content varied from 2 mg kg^{-1} to 198 mg kg^{-1} dry matter. Results from HPLC analysis of the samples showed that gallic acid and (+)catechin hydrate were the major phenolic compounds in both whole grapes and pomace. Cyanidin and petunidin 3-*O*-glucoside were the major anthocyanins in the juice.

Introduction

Cynthiana (also known as Norton) is a variety of grape (*Vitis aestivialis*) that is native to North America and is renowned for its ability to produce an intense-colored red wine. The local regions where Cynthiana is produced are Arkansas, Illinois, Indiana, Kansas, Louisiana, Maryland, Missouri, Oklahoma, New Jersey, Tennessee, Texas, Virginia,West Virginia, and North Carolina (Roberts 1999).

Phytochemicals are important compounds found in plants. Their importance is based on their biological activities and health promoting benefits (Detre and others 1986; Balentine 1999). Phenolic compounds are a subdivision of this group. Recently, phenolic compounds have attracted passionate interest of both researchers and consumers due to their antioxidants and antimicrobial activities (Thimothe 2007b; Rodríguez-Montealegre 2005; Puupponen-Pimiä 2001; Negro 2003). The antioxidant activities of phenolic compounds are attributed to their free radical scavenging and metal chelating properties, as well as their effects on cell signaling pathways and on gene expression (Yilmaz 2006; Muñoz-Espada 2004; Jayaprakasha 2001; Hogan 2009; Heinonen 2003). The mechanism is mainly influenced by the number of OH groups and their position on the ring in the

molecule, which determines the antioxidant capacity of phenolic compounds (Hogan 2009; Heinonen 2003; Detre 1986; Balentine 1999). Several studies have showed that the antimicrobial activity of phenolic compounds is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, more lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya 1996b). Other mechanisms of action noted by the study of phenols in general and subclasses of phenolic acids, flavonoids and tannins involve enzyme inhibition, enzyme inactivation, formation of complexes with cell walls and metal ions (Cowan 1999). The phenolic compounds are cyclic benzene compounds with a minimum of one hydroxyl group associated directly with the ring structure. Based on their structure two groups are distinguished flavonoids and non-flavonoid phenols (Bowyer 2002a). Flavonoid phenols are subdivided into anthocyanins, flavanols, flavonols and tannins (Kennedy 2001; Allen 1998). Non-flavonoid phenols consist primarily of phenolic acid and esters (Singleton 1976). The differences between both groups are the number and orientation of phenolic-subunits with the molecules (Bowyer 2002a).

Flavonoids are widely distributed in grapes, especially in seeds and stems, and principally contain (+)-catechins, (−)-epicatechin. Anthocyanins are pigments and mainly exist in red grape skins. Phenolic acids in grapes comprise derivatives of hydroxycinnamic acid, including caffeic acid and p-coumaric acid (Rodríguez-Montealegre 2005; Kammerer 2004b). Ozimina found that either phenolic acids or the anthocyanin profiles seem to be closely related to the variety of grape (Ozimina 1979). Nevertheless, other studies have evidenced variations in the occurrence of flavonoids with seasonal conditions or viticultural practices (Ryan and Revilla 2003; Roberts 1999).

Most of the research on phenolic compounds has been focused on the structural characterization of non-flavonoid/flavonoid phenols from a wide variety of plant matrices. Fruits and berries contain a variety of phenolic compounds, which are often located in the external layer of the plant, seeds and pulp and are readily extracted by organic solvents (Heinonen 2003). The external location of phenolic compounds is associated with their main natural function: protection of the plant against environmental stress and pathogens.

The extraction of phenolic compounds is primarily influenced by sample particle size, the extraction method, and storage time (Thorsten Maier 2008). Additional steps may be required for the removal of unwanted non-phenolic substances such as waxes, fats, terpenes and chlorophylls. Solid phase extraction (SPE) techniques and fractionation based on acidity are commonly used to remove non-phenolic substances (Rodríguez 2000). For extraction of phenolic compounds, soxhlet extraction is one of the most popular techniques for isolating non-flavonoid/flavonoid phenols from solid samples. This is probably due to its simplicity, inexpensive extraction apparatus and its use in the EPA official methods (Morales 2000). Polar solvents, such methanol, acetone, acetonitrile yield high extraction efficiencies. However, they also extract other undesirable polar compounds present in samples (Alonso 1998). With apolar solvents, such as n-hexane or dichloromethane, the extraction of phenolic compounds occurs after an acid digestion step. Satisfactory recoveries of phenols from soils and sediments have been reported with this technique using, in most cases, mixtures of polar and apolar organic solvents (Rodríguez-Montealegre 2005). Alonso (Alonso 1998) developed an analytical soxhlet protocol for the determination of priority phenolic compounds in soil

samples using a solvent mixture, methanol-water (4:1) containing 2% triethylamine, to enhance the extraction of more chlorinated phenols. Recoveries varied in the range from 67-97% with a standard deviation between 8 and 14%. Despite, the results obtained with this methodology, Soxhlet extraction makes the analysis procedure very time consuming.

Reversed-phase HPLC using photodiode array detection has been extensively reported for the identification and quantification of phenolic compounds in grapes and wine (Gao 1995b). Nevertheless, most of these methods target only certain classes of phenolic compounds (example: flavonoids and stilbenes vs. anthocyanins) whereas, simultaneous determination of all compounds is fairly unusual (Lin 2007).

The objectives of the current study were: (a) to develop a single HPLC protocol for quantification of both non-flavonoid and flavonoid phenols in Cynthiana grape, (b) To identify and quantify the major phenolic compounds from the quantitative extracts of Cynthiana whole grape, juice, and pomace, and c) To identify and quantify the major phenolic compounds in Cynthiana whole grapes, juice, and pomace using the following crude extraction conditions: 50% methanol-water mixture, 70% methanol-water mixture, 50% acetone-water mixture, 100% petroleum ether and 0.01% pectinase solutions.

Materials and Methods

Solvents and Reagents. Methanol, acetonitrile, acetone, petroleum ether, and phosphoric acid were purchased from Fisher Scientific (Fair Lawn, NJ and were of analytical or HPLC grade. Water was from Milli-Q purification system Millipore (Millipore, Bedford, MA, USA). Ascorbic acid, ethyl acetate and β- glucuronidase type HP-2 from *Helix pomatia* were purchased from sigma Aldrich (St Louis, MO, USA. Gallic acid, Ferulic acid, Caffeic acid, p-coumaric, (+)

Catechin hydrate, Quercetin, (-) Epicatechin gallate, Isorhamnetin, Myricetin, trans-resveratrol, 7-ethoxycoumarin and β-glucosidase were purchased from Fluka (St Louis, MO. USA); The 3-*O*- glucosides of delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin were obtained from Polyphenols Laboratories AS (Sandnes, Norway). Sep-Pak C_{18} cartridges (1g, 6 mL) were obtained from Waters Corporation (WAT051910, Waters Corp., Milford, MA, USA).

Grape collection. Thirty-five pounds of Cynthiana grape (clusters) were collected from a Cimmaron Valley Research Station Center at Oklahoma State University (Perkins, OK). Clusters were placed in 7 different bags (~5 lbs) and vacuum packed (16 x 25 inch vacuum bags, Curwood, Inc, Oshkosh,WI) under low vacuum (100 KPa to 3KPa, Multivac C500, Multivac Inc. Kansas City, MO) and stored at -20°C until future analysis.

Pomace preparation. Wine was made in order to collect Cynthiana pomace because of the difficulties in obtaining commercial pomace at the time the study was conducted. Cynthiana pomace was produced on pilot-scale level by the following protocol: approximately 30 pounds of frozen grapes were weighed and thawed at 4 ºC for two days. Grapes were destemmed and gently crushed on with a commercial grape destemmer-crusher (Jolly-60, St. Patrick's of Texas, Austin, TX). They were placed into a 100 liter (25 gallon) stainless steel fermentation vessel for maceration and wine grade yeast (*Saccharomyces cerevisiae*) and yeast nutrient (Fermaid) was also added. The vessel was capped with adjustable height lids allowing approximately 25cm (≈10 inches) of headspace at 20-22°C for 5 days in order to mimic industrial process. Samples were monitored by rapid residual sugar tests (AV-RS Accuvin LLC, Napa, CA). The lid was pressed down to minimize head space 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 wine and pomace (Jensen 2008b). Samples of

pomace were collected in vacuum bags (16 x 25 inch vacuum bags, Curwood, Inc, Oshkosh, WI) and stored at -20°C until further analysis. Wine samples were discarded.

Preparation of whole grapes, juice and pomace for extraction. For whole grapes, woodchip and stems were removed from approximately 5 lbs of frozen clusters. In order to create a frozen powder from the grapes, whole grapes were placed in liquid nitrogen (-196°C) using a metal strainer. Liquid nitrogen treated grapes were ground for 30 s in a 4°C room using a Waring blender (model 51BL31) and a previously frozen blender jar. The resultant powder was placed in vacuum bags (8 x 10 inch vacuum pouches, Mid-Western Research & Supply, Inc). Prior to vacuum packaging and frozen storage, a subsample was collected for immediate extraction and analysis.

For pomace, liquid nitrogen powdering and subsampling was conducted as described for whole grapes.

For juice, 454 grams of grapes were pressed by hand using cheese-cloth. Juice was added to an amber vial (530 mL Glass Amber with Teflon face lined cap, Fisherbrand, ThermoFisher Scientific Inc.) and kept at 4°C. Analysis was conducted the same day.

Selection of extraction temperature. Preliminary experiments were conducted to determine optimum temperature for extraction for selected protocols (Vassan 2009; Torres 2005b; Kammerer 2004b). The following extraction conditions were evaluated: ice bath, room temperature and 40°C (data not shown). Results indicated extraction on ice maximized phenolic recovery. Each extraction was carried out in triplicate and each extract was injected in duplicate.

Quantitative Extraction Protocols

Quantitative extraction I (phenolic acids, flavonols, flavonols, and stilbenes). This extraction was modified from Torres (Torres 2005b). Juice was first pre-treated with HCl in order to hydrolyze sugar whereby the final concentration of HCl in juice was 0.1%. Treated juice was centrifuged for 3,000 x g for 15 min prior to sampling.

Briefly, 0.5 g of sample (whole grape powder or pomace powder or pre-treated juice) was weighed (A-160, Denver Instruments Co) and transferred to a 30 mL brown bottle (Glass Amber with Teflon face lined cap, Fisherbrand, ThermoFisher Scientific Inc.) and 25 μL of 25 ppm 7-ethoxycoumarin (internal standard) and 4 mL of 50% v/v methanol-water mixture were added. The bottle was placed in an ice bath and the mixture was stirred for 1 h. The mixture was subsequently centrifuged (Clinical 50-82013-800 centrifuge VWR International, Chicago) at 3,000 x g for 20 min and decanted through whatman filter paper (#41) into a 10 mL volumetric flask. Samples were then re-extracted under the same conditions and the combined filtrates were brought to volume with 10 mL of 50% methanol-water mixture. An aliquot of 2 mL was placed into a brown vial (3 mL Glass Amber with Teflon face lined cap, Fisherbrand, ThermoFisher Scientific Inc), to which 110 μL of 0.78M acetate buffer (pH 4.8), 100 μL of ascorbic acid and 50 μL of β-glucuronidase were added. Vials were capped, vortexed and incubated at 37°C for 17 h (overnight). Samples were centrifuged at 4,000 x g for 25 min and analyzed by RP- HPLC (Thimothe 2007b).

Quantitative extraction II (flavonoid-anthocyanins). The extraction was adapted from Kammerer (17) with some modifications. Briefly, 5 grams of sample, 200 μL of the internal standard 25 ppm 7-ethoxycoumarin and 100 mL of methanol/0.1% HCl (v/v) were combined and

mixed in a brown bottle for 1 h under stirring and flushing with nitrogen in order to prevent oxidation during extraction at room temperature. The extract was centrifuged at 4,000 x g for 10 min, and the material was re-extracted with 100 mL of the organic solvent under the same conditions for 15 min. A 5 mL aliquot of the combined supernatants were evaporated to dryness under nitrogen in a water bath (Zymark TurboVap, Zymark Center, Hopkinton, MA) at 30°C to remove the organic solvent and the residue was then dissolved with 2 mL of acidified water (pH 3.0, acetic acid). Anthocyanins were analyzed by RP-HPLC (Thimothe 2007b).

Preparation of crude extracts. The extraction conditions (time, solvent to solid ratio, temperature) were based upon literature data (Ju 2003) and previous extraction experiences of the research group (Vassan 2009). Conditions during all crude extraction experiments were: solvent-to-ratio of 40 mL solvent per 20 gram extraction material and extraction time 1 h. The solvents were removed by nitrogen evaporation at 35°C. The extraction solvents used were: 70% methanol-water, 50% acetone-water, 0.01% pectinase- water mixture and petroleum ether.

Crude extraction of the polyphenols from whole grape or pomace powder was conducted by weighing 20 g of sample into 125 mL erlenmeyer flasks. The appropriate solvent, 40 mL, was added. The flasks were placed in a shaker (Classic C76, New Brunswick Scientific, Edison, NJ) maintained at 18°C and 250 rpm for 1 h. After shaking, samples were filtered under vacuum using a Buchner funnel with 5.5 cm diameter (55mm #1, Whatman Inc. Ltd., Mainstone, England). Samples were filtered until there was no visible dripping and then rinsed twice with approximately 10 mL of solvent for two subsequent filtrations. The final filtrates (except petroleum ether extracts) were transferred to 100 mL volumetric flask and brought up to volume with the corresponding solvent. The petroleum ether extracts were allowed to evaporate and were re-suspended in 100% acetone. A 10 mL aliquot was subsequently evaporated to dryness

with nitrogen a water bath at 30°C, dissolved in 7 mL of Milli-Q water, and applied to solid phase extraction cartridges (WAT051910, Waters Corp., Milford, MA). Aliquots of 5 mL were applied to the cartridges, which were activated with 5 mL of methanol, rinsed with 5 mL of deionized water and 3 mL of 0.01% HCl (v/v). Samples were eluted with 5ml of methanol and filtered through 0.45µm nylon filters (Fisherbrand, PTFE, Fisher Scientific, Denver, CO) and used for RP-HPLC analysis (Thimothe 2007b).

RP-HPLC analysis. The RP-HPLC procedure utilized was modified from Timothe (2007b). The method was designed to separate 17 phenolic compounds. Phenolic acids, flavanols, flavonols, stilbenes (PAFFS), standards were received as individual compounds. Flavanoid-anthocyanin (FA) standards were received as a mixture. A 100 ppm standard solution containing all (11) of the individual PAFFS, in addition to the FA mixture (6) was prepared. This standard solution also contained 25 ppm internal standard (7-ethoxycoumarin). The standard curve was prepared by serially diluting (1:1) to a final concentration of 0.78 ppm. Separation, identification and quantification of individual phenolic compounds were performed on a reversed phase chromatography system (Alliance Waters 2690, Waters, Ireland) with a photodiode array detector (PDA, Waters 2996) and Empower 2 software (waters). Compounds were separated by a gradient elution system on a Sun Fire™ C18 column (5µm particle size, 4.6 x 250 mm i.d.) including a guard column (5µm particle size, 4.6 x 30 mm) at 25°C. The flow rate was set to 1.0 mL/min. For gradient elution, mobile phases A and B were employed. Solution A contained 0.1 % H_3PO_4 in MilliQ water, and solution B contained 0.1 % H_3PO_4 in acetonitrile (HPLC grade). Data acquisition was applied for 45 min with a total run of 65 min. Gradient elution was as follows: 92% A/8% B, at 0 min; 85% A/15% B at 5 min; 40% A/60% B at 45 min; 40% A/60% B at 55 min; and back to initial conditions 92% A/8% B at 60 min. The

PDA was set at 210-600 nm and chromatograms were extracted at 280, 320, 370 for phenolic acids and flavonoids, and 520 nm for anthocyanins.

Statistical analysis. Data were analyzed using ANOVA to determine differences among solvent means using PROC GLM of Statistical Analysis SAS 9.2 (Cary, NC) version 9.2. (SAS Inst. 2003). Experimental designed was 5 x 2 x 2 factorial in a completely randomized design comparing five organic solvents (50% Methanol, 70% Methanol, 50% Acetone, 0.01% Pectinase and petroleum ether) evaluated in two different group (whole grape and pomace) each with two analytical replicates. Juice sample was not analyzed using the experimental design described above because the extraction with the different solvents was not prepared. Means were separated by Tukey's ($P < 0.05$). All experiments were conducted in triplicate.

Results and Discussion

Selection of Quantitative Extraction Protocol

Cynthiana grapes analyzed in this study were selected because they represent an important cultivar for red winemaking in Oklahoma. Red wine grapes were chosen due to their higher phenolic content compared to white/table grapes and because of the availability of pomace as an inexpensive source of extractable material. Phenolic compounds were extracted from grapes and pomace using different organic solvents.

RP-HPLCanalysis. Several factors such as maximum absorbance, retention time, mobile phases and concentration were studied to develop a method capable of resolving a large number of the phenolic compounds that are present in grape. This method differed from Timothe (2007b) by increasing the amount of the organic mobile phase (B) from 11% to 15% at 5 min and maintaining that amount of the organic mobile phase in isocratic elution for 40

minutes. This change allowed all the phenolic compounds investigated in this study to be eluted using a single HPLC method. **Figure 1** represents a typical separation chromatogram of the standards at different wavelengths.

Quantitative extraction I (phenolic acids, stilbenes, and flavonols). In order to evaluate the effectiveness of the crude extractions on whole grape, juice and pomace, data was collected from a "quantitative" extraction. Quantitative extraction of phenolic compounds from grape has been previously reported (Wulf 1978; Thimothe 2007b; Kammerer 2004b). The extractions typically include the utilization of enzymatic hydrolysis to simplify chromatographic data. The Enzyme β-glucuronidase from *H. pomatia* Type-HP-2 is used to cleave the sugar moiety off of phenolic glycosides (Yáñez 2007). In addition, it was reported that β-glucuronidase contained arylsulfatase activity and can also effectively deconjugate flavonoid glucosides in red fruits (Thompson 2008).

The levels of individual and total PAFFS (phenolic acid, flavanols, flavonols and stilbenes) and FA (flavonoid-anthocyanids) measured in grape juice; whole grape and pomace are displayed in **Tables 1, 2,** and **3** respectively. In juice, 6 of 11 PAFFS were recovered. However, for whole grape only 3 PAFFS were recovered. For pomace, 9 of 11 PAFFS were recovered. The highest recovered PAFFS compound in whole grape was (+)-catechin hydrate. It was also the highest recovered in pomace, but at a level that was more than 4 times lower than in whole grape sample. The PAFFS concentration of the analytes measured in juice was 7 mg/kg dry matter. The results obtained in the present study were comparable with previous studies by Stalmach (Stalmach 2011), who found concentrations of PAFFS and FA in grape juice of 8.4 mg/g and 680 mg/g. PAFFS concentration of the analytes measured in whole grape was 1025 mg/kg dry matter, which they are in agreement with previous results found in Cynthiana grape

(Rivera-Dominguez 2010; Reisch 1993). In the whole grape and pomace PAFFS were the major phenolic compounds accounting for 74 and 86%, respectively all phenolic compounds measured. In contrast, PAFFS only comprised a minor proportion (<1%) of total phenolic compounds in grape juice. The PAFFS concentration of the analytes measured in pomace was 391 mg/kg dry matter, which is in agreement with the results found by Hogan (Hogan 2010), who reported total phenolic composition of 475.5 mg/g dry matter in Cynthiana grape pomace extract. **Figure 2** shows a typical separation of PFFAS in grape pomace.

Quantitative extraction II (flavonoid-anthocyanins). Anthocyanins are found in nature as anthocyanidin glycosides or acetylated glycosides and they are usually extracted from plant materials with an acidified organic solvent, most commonly methanol. In the past, several studies have classified more than fifteen anthocyanidin glycones (Harborne and Williams 2000). In this study only six of them were identified and corresponded to cyaniding 3-*O*-glucoside (Cy3G), delphinidin 3-*O*-glucoside (Dp3G), malvidin 3-*O*-glucoside (Mv3G), pelargonidin 3-*O*-glucoside (Pg3G), peonidin 3-*O*-glucoside (Pe3G), and petunidin 3-*O*-glucoside (Pt3G) present in red fruits. Acidic solvents can cause the hydrolysis of glucoside groups joined to flavonoids, thus enhancing their migration into the solvent (Mazza 1995).

In juice, FAs were the most abundant phenolic compounds recovered by this extraction technique. The main FAs quantified in this study were Mv3G, Pe3G, De3G, Pg3G and Pt3G. The FA concentration of the analytes measured in juice was 1808 mg/kg dry matter. **Figure 3** shows a typical separation of anthocyanins in juice (A) at 520 nm.

The recovery of FA was higher than for PAFFS in whole grape. Thus, the use of methanol/0.1% HCl as a solvent for whole grape resulted in a significantly higher extraction of

FA than for any other solvent evaluated ($P < 0.05$) (**Table 2**). The concentration of FA using this is extraction method was 362 mg/kg dry matter. The results of FA concentration were similar to studies using Cynthiana grape by Cho (Cho 2004), who reported to have 358 mg/kg of total anthocyanins in Cynthiana grape. The higher efficiency of methanol/ 0.1 % HCl is a consequence of the association of phenolic compounds with cell wall polymers and could be partially explained by the capacity to degrade cell walls and seeds, which have unpolar character and cause phenolic compounds to be released from cells (O'Neil 2006). **Figure 3** shows a typical separation of FA in whole grape (B) at 520 nm.

As can be noticed in grape pomace, the recovery of FA was lower than for PAFFS. Hogan found that total anthocyanins were lower than total phenolics in Cynthiana (*Vitis aestivalis)* with values of 0.93 C3GE (Cyanidin 3-glucoside equivalent) mg/g and 1.82 GAE (Gallic acid equivalent) mg/g respectively (Hogan 2010). **Figure 3** shows a typical separation of anthocyanins in grape pomace (C) at 520 nm. The whole grape was detected to have the highest recovery of FA (362 mg/kg dry matter) than grape pomace (80 mg/kg dry matter) ($P > 0.05$). These differences suggest a potential impact of winemaking process on FA content.

Crude extracts. Extraction of phenolic compounds from grape using acetone: water, methanol: water and water has been previously reported (Revilla 2003; Lapornik 2005; Ju 2003). The effectiveness of crude extractions in recovering PAFFS and FA from whole grape and pomace was measured by comparing results to the previously described quantitative extractions. Significant differences were found among the solvents used for extraction of phenolic compounds in whole grape ($P < 0.05$) (**Table 2**). In whole grape, all crude extracts produced a higher recovery of FA than for PAFFS. The concentration of FA using petroleum ether showed

the highest recovery 285 mg/kg dry matter. This solvent was also the most efficient recovering PAFFS (86 mg/kg dry matter).

As can be noticed in grape pomace, significant differences were found among the solvents ($P < 0.05$) (**Table 3**). The highest recovery of FA and PAFFS were observed using 50% acetone and 0.01% pectinase. The highest concentration of FA and PAFFS using acetone was 22703 mg/kg and 3110 mg/kg dry matter respectively. Yilmaz and Toledo (Yilmaz 2006) compared methanol, ethanol and acetone water mixtures for extracting phenolic compounds from grape pomace, and they found recoveries of phenolic compounds were higher using acetone. Extractions of FA and PAFFS using 0.01% pectinase yielded to concentration of 22076 mg/kg and 2840 mg/kg dry matter respectively. **Figure 3** shows a typical separation of anthocyanins in grape pomace (C) at 520 nm.

Comparison of solvent recovery of phenolic compounds on whole grape and pomace. Several researchers have used organic solvent-water mixture for the extraction of the phenolic compounds in red grapes (Negro 2003; Martínez Vidal 2004; Bonilla 1999b) but only few of them have directly compared the ability of different solvents to recover phenolic compounds (Pinelo 2005; Lapornik et al. 2005; Ju 2003). Lapornik, Prosek and Wondra compared 70% methanol and water for extracts prepared by plant by-products (Lapornik et al. 2005) and they found that 70% methanol was the most effective on recovery. Vatai and Knez compared different concentrations of acetone, ethyl acetate, and ethanol for extracting phenolic compounds from grape and they found that 50% acetone was the most effective for recovery.

The Quantitative extraction I (QI) and Quantitative extraction II (QII) of whole grapes recovered more PAFFS and FA than all other solvents evaluated (**Table 2** and **3**; $P < 0.05$).

However, this was not the case for the pomace extraction. In fact, both the QI and QII extractions of pomace performed poorly in the recovery of phenolic compounds. There was no difference in the efficiency of the crude extracts to recover phenolic compounds from whole grapes and their poor recoveries suggest they are not good alternatives to QI and QII for this particular application. However, this is not the case for their application on pomace. With pomace, QI and QII showed very low recoveries of phenolic compounds. On the other hand, all crude extract solvents were superior in recovering phenolic compounds from pomace than QI and QII. The recovery of PAFFS and FA from pomace (**Table 3**), 50% acetone and 0.01% pectinase were most effective ($P < 0.05$). The hypothesis to explain our results is referred to the Hansen solubility parameter values, which are based on δD (dispersion bonds). Acetone and water have high δD both with values of 15.5. A polar solvent such as water tends to charge negatively solutes via hydrogen bond and acetone tends to have a large dipole moment (separation of partial positive and partial negative charges within the same molecule) and dissolution positively charged species via their negative dipole. The values of dipole moment for water and acetone are 1.85D and 2.88D respectively; and the values for methanol and petroleum ether are 170D and 1.15 respectively (Hansen 2000). Neither methanol nor petroleum ether was as effective as the 50 % acetone and 0.01% pectinase. The PAFFS and FA values of extracts when using 50% acetone solvent was the highest among the other solvents (3605 mg/kg and 22254 mg/kg, dry matter). Other researchers have reported that grape pomace not only has a high content of FA compared to the other samples, but also appears to have a higher content of unknown compounds (Thimothe 2007b). Similar observations were made in the current study (Figure 3). These unknown peaks were also detected on whole grape sample and can be related to acylated anthocyanins, which are present in abundance in grapes (Hong 1990).

Conclusion

In the present study, identification and quantification of the major phenolic acids, flavonoids and anthocyanins present in Cynthiana were established by chromatographic profiles for whole grape, juice, and grape pomace. The results confirm that Cynthiana grape and its by-products are potentially sources of natural phenolic compounds. The type of solvent used for extraction affected quantity and composition of phenolic compounds in whole and grape pomace extracts. It was generally observed for grape pomace sample that by using 50% acetone: water as a solvent, the highest yields of phenolic compounds were obtained. Results indicate that the utilization of 0.01% pectinase: water mixture, an economical and natural solvent, was also effective for isolating phenolic compounds from grape pomace sample. Chemical analysis, including characterization of the acylated anthocyanins, high molecular weight proanthocyanidins are needed on the extracts in order to evaluate biological activities of phenolic compounds from Cynthiana.

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Table 1. Content of phenolic compounds identified in grape juice (mg/kg ± RSD) when extracted quantitatively. Results are reported on a dry matter basis. PAFFS = phenolic acids, flavonols, flavanols and stilbenes. FA = Flavonoid-anthocyanins. Data are the mean for three replications.

	Analyte	**Juice**
PAFFS	(-) Epicatechin gallate	0.34 ± 0.09
	(+) Catechin hydrate	6.06 ± 0.17
	Caffeic acid	0.07 ± 0.01
	Ferulic acid	0.35 ± 0.15
	Gallic Acid	0.44 ± 0.26
	Isoharmnetin	< 0.1
	Kaempferol	< 0.1
	Myricetin	< 0.1
	p-coumaric acid	0.15 ± 0.09
	Quercetin	< 0.1
	Resveratrol	< 0.1
	TOTALS	7.41 ± 0.97
FA	Cy3G	910.09 ± 0.34
	Dp3G	17.22 ± 0.15
	Mv3G	194.82 ± 0.06
	Pe3G	118.52 ± 0.41
	Pg3G	67.88 ± 0.26
	Pt3G	499.91 ± 0.17
	TOTALS	1808.45 ± 140.23

Table 2. . Content of phenolic compounds identified in whole grape (mg/kg ± RSD) when extracted quantitatively. Results are reported on a dry matter basis. PAFFS = phenolic acids, flavonols, flavanols and stilbenes. FA = Flavonoid-anthocyanins. Data are the mean for three replications.

	Analyte	50% Acetone	70 % Methanol	0.01% Pectinase	Petroleum Ether	Quantitative extraction I
PAFFS	(-) Epicatechin gallate	12.52 ± 0.92	< 0.1	< 0.1	< 0.1	< 0.1
	(+) Catechin hydrate	18.88 ± 0.76	20.79 ± 0.07	55.32 ± 0.27	53.80 ± 0.06	875.83 ± 0.21
	Caffeic acid	2.28 ± 0.46	7.89 ± 1.02	< 0.1	3.18 ± 0.14	< 0.1
	Ferulic acid	13.06 ± 1.56	8.11 ± 1.09	5.00 ± 0.64	15.26 ± 0.02	< 0.1
	Gallic Acid	< 0.1	7.20 ± 0.06	5.46 ± 0.28	< 0.1	115.90 ± 0.12
	Isoharmnetin	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
	Kaempferol	1.50 ± 0.01	1.73 ± 0.02	< 0.1	1.78 ± 0.08	< 0.1
	Myricetin	0.88 ± 0.01	1.43 ± 0.15	< 0.1	0.98 ± 0.18	< 0.1
	p-coumaric acid	8.18 ± 0.58	15.18 ± 1.27	2.66 ± 0.83	10.40 ± 0.02	< 0.1
	Quercetin	2.63 ± 0.06	< 0.1	1.04 ± 0.09	0.67 ± 0.09	33.74 ± 0.18
	Resveratrol	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
	TOTALS	59.76 ± 2.24[c]	62.35 ± 2.64[c]	69.48 ± 9.48[c]	86.09 ± 0.37[b]	1025.49 ±268.05[a]
FA	Cy3G	29.88 ± 0.09	23.50 ± 0.34	28.25 ± 1.26	2.74 ± 0.39	68.58 ± 0.43
	Dp3G	8.04 ± 0.09	< 0.1	8.09 ± 0.33	1.69 ± 0.50	46.88 ± 0.86
	Mv3G	2.56 ± 1.07	3.43 ± 0.27	9.16 ± 1.28	3.27 ± 0.73	3.69 ± 0.56
	Pe3G	< 0.1	< 0.1	< 0.1	< 0.1	45.67 ± 0.32
	Pg3G	26.08 ± 0.16	< 0.1	< 0.1	3.55 ± 0.04	4.07 ± 0.11
	Pt3G	53.60 ± 0.63	88.99 ± 0.96	228.36 ± 0.66	273.93 ± 0.03	192.73 ± 0.48
	TOTALS	120.91 ± 8.32[b]	115.93 ± 25.83[b]	273.88 ± 53.50[b]	285.19 ± 54.22[b]	362.48 ±28.49[a]

[a-c] Means with similar letter are not significantly different (Tukey, $P > 0.05$)

Table 3. Content of phenolic compounds identified in grape pomace (mg/kg ± RSD) when extracted quantitatively. Results are reported on a dry matter basis. PAFFS = phenolic acids, flavonols, flavanols and stilbenes. FA = Flavonoid-anthocyanins. Data are the mean for three replications.

	Analyte	50% Acetone	70 % Methanol	0.01% Pectinase	Petroleum Ether	Quantitative extraction I
PAFFS	(-) Epicatechin gallate	378.14 ± 0.76	80.94 ± 0.54	44.92 ± 0.27	103.05 ± 0.15	< 0.1
	(+) Catechin hydrate	1738.89 ±0.87	356.01 ± 1.56	2293.45 ± 0.40	98.87 ± 0.21	198.84 ± 0.07
	Caffeic acid	438.43 ± 0.54	33.40 ± 1.05	198.50 ± 0.66	36.68 ± 0.47	< 0.1
	Ferulic acid	1.33 ±0.87	< 0.1	0.27 ± 0.19	< 0.1	2.72 ± 0.03
	Gallic Acid	95.36 ± 0.61	7.38 ± 0.48	118.66 ± 0.28	93.87 ± 0.18	115.94 ± 0.13
	Isoharmnetin	131.51 ± 1.20	2.79 ± 0.34	54.21± 0.71	67.36 ± 0.84	14.02 ± 1.64
	Kaempferol	28.53 ± 1.02	3.63 ± 0.49	52.23 ± 0.51	53.25 ± 0.56	27.50 ± 0.08
	Myricetin	36.77 ± 1.02	23.40 ± 0.96	14.64 ± 0.28	34.76 ± 1.18	8.43 ± 0.01
	p-coumaric acid	214.55 ± 0.73	24.11 ± 0.59	22.19 ± 0.45	42.77 ± 0.50	3.20 ± 0.18
	Quercetin	26.25 ± 0.86	3.06 ± 1.10	30.54 ± 0.38	60.08 ± 0.76	18.55 ± 0.64
	Resveratrol	20.66 ± 0.89	5.60 ± 0.37	11.27 ± 0.27	36.58 ± 0.64	2.05 ± 0.06
	TOTALS	3110.43± 152.41[a]	540.31± 34.39[b]	2840.89± 204.25[a]	627.29 ± 8.54[b]	391.26 ± 22.78[b]
FA	Cy3G	10013.29 ± 1.44	489.63 ± 0.95	8510.22 ±0.29	84.22 ± 0.23	14.34 ± 0.11
	Dp3G	2648.69 ±0.98	324.46 ± 1.25	422.51 ± 0.68	189.00 ± 0.58	25.45 ± 0.09
	Mv3G	7289.32 ± 1.05	502.94 ± 1.46	2055.25 ± 0.25	101.68 ± 0.69	21.55 ± 0.03
	Pe3G	922.74 ± 1.08	97.76 ± 1.25	333.04 ± 0.42	120.71 ± 0.54	5.37 ± 0.18
	Pg3G	219.16 ± 0.97	38.70 ± 1.34	114.80 ± 0.21	36.33 ± 0.41	2.35 ± 0.01
	Pt3G	1610.64 ± 1.06	105.90 ± 1.42	10640.45 ± 0.28	93.95 ± 0.45	6.56 ± 0.08
	TOTALS	22703.85± 9268.81[a]	1559.42 ± 84.64[b]	22076.28 ± 1905[a]	625.89 ± 20.47[b]	80.90 ± 4.03[c]

[a-c] Means with similar letter are not significantly different (Tukey, $P > 0.05$)

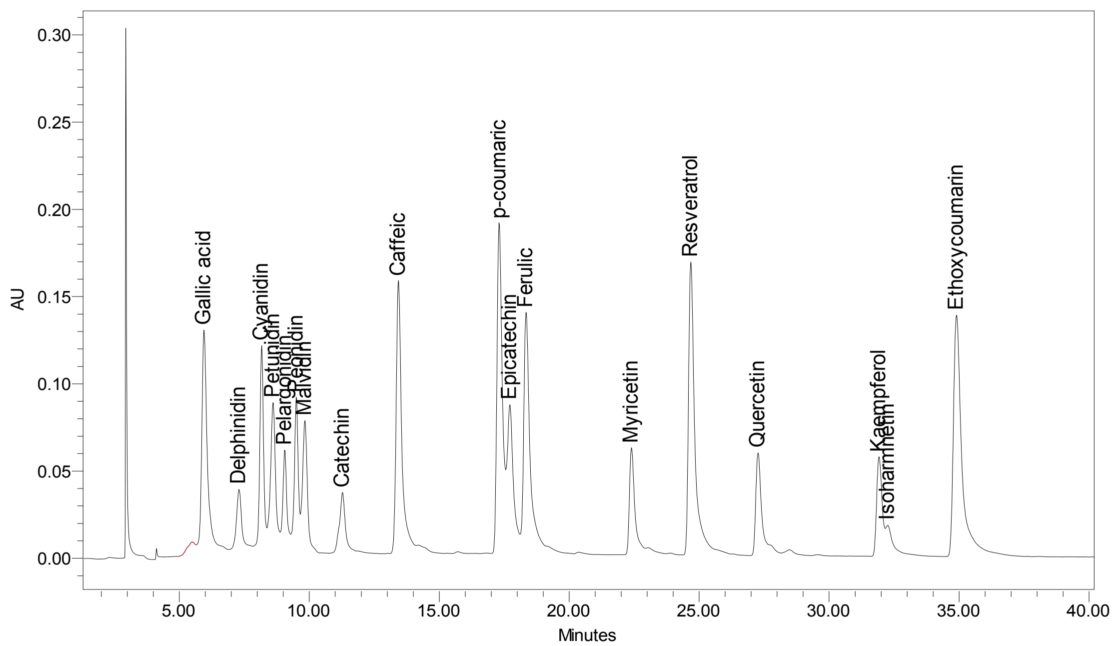
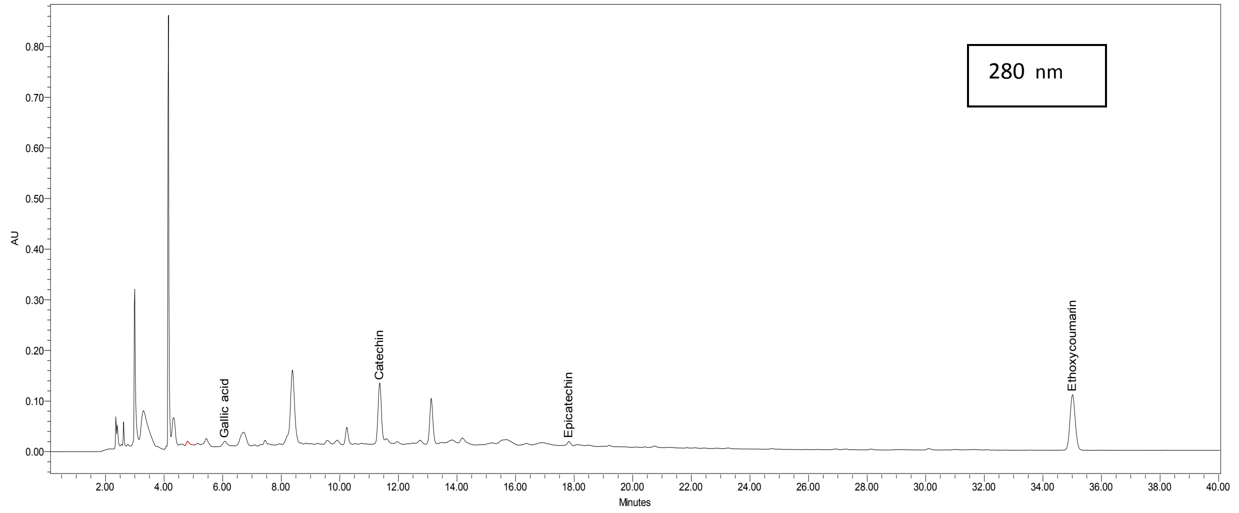
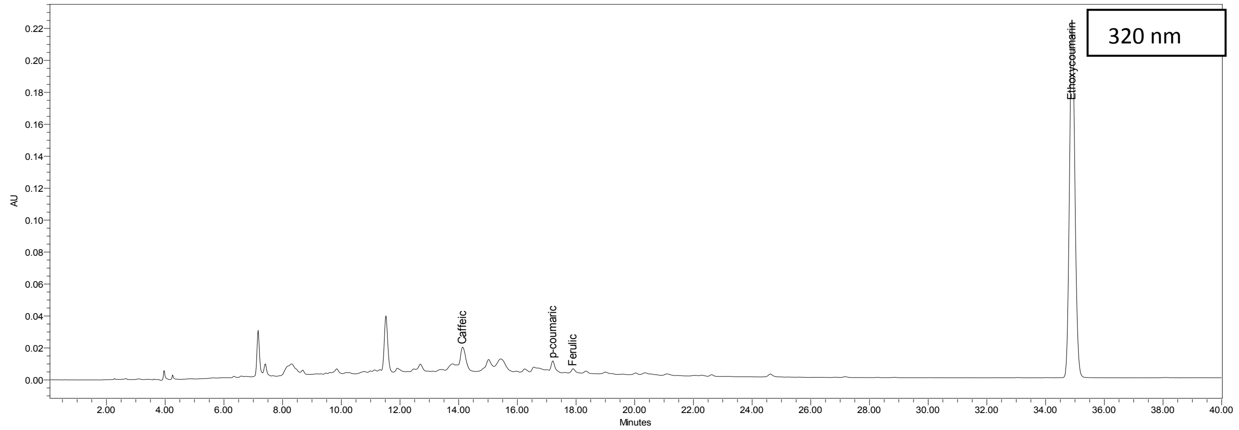
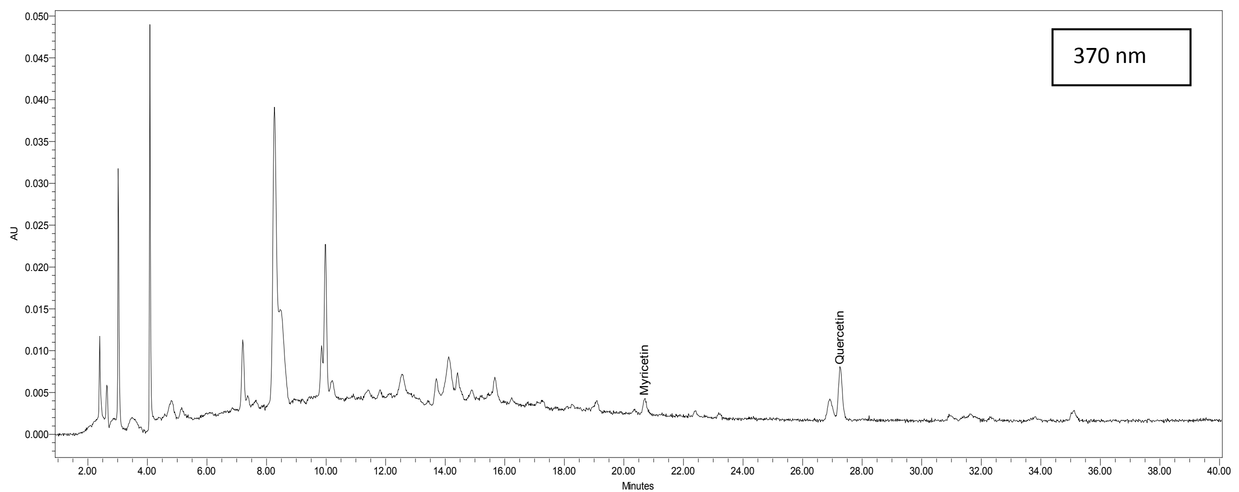


Figure 1. Typical separation chromatogram of standards mixture concentration of 50 ppm

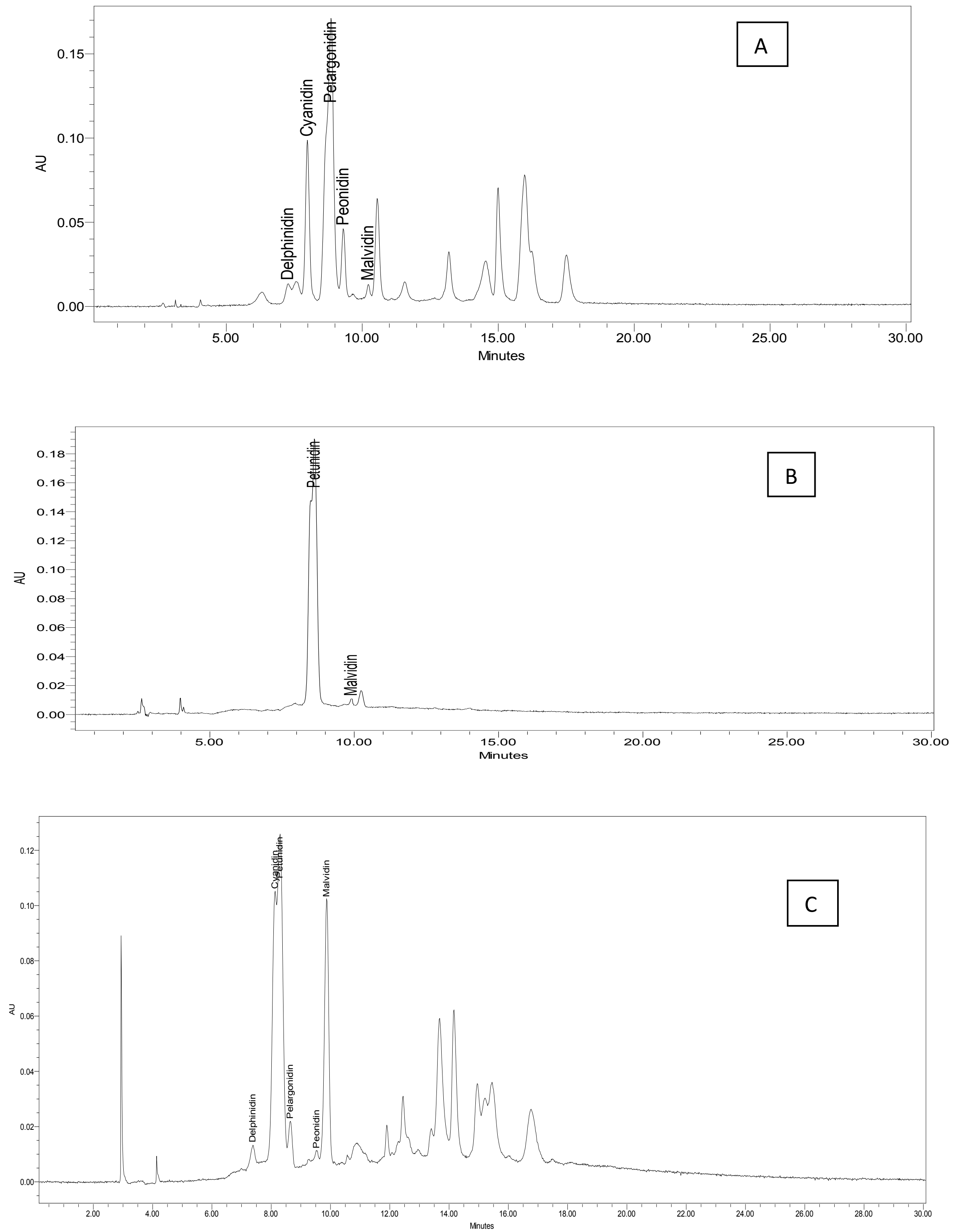






HPLC-PDA chromatogram (280, 320, 370 nm) of the predominant phenolic compounds present in grape pomace using enzymatic hydrolysis.

Figure 2. Typical separation of phenolic, flavonol, flavanol and stilbene compounds in grape pomace



HPLC-PDA chromatogram (520 nm) of the predominant anthocyanins compounds present in (A) whole grape, (B) Juice, and (C) grape pomace

Figure 3. Typical separation of anthocyanins in whole grape, juice and grape pomace at 520 nm.

CHAPTER IV

ANALYSIS OF PHENOLIC COMPOUNDS IN COMMERCIAL DRIED GRAPE POMACE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Abstract

Phenolic acids, flavanols, flavonols and stilbenes (PAFFS) were isolated from commercial dried grape pomace and purified using enzymatic hydrolysis. Flavonoid-anthocyanins (FA) were isolated using methanol/0.1% hydrochloric acid extraction. In addition, crude extractions of phenolic compounds from pomace using 50% methanol, 70% methanol, 50% acetone, 0.01% pectinase, or petroleum ether were also evaluated. Reverse phase high performance liquid chromatography (RP-HPLC) with diode array detector was used to identify phenolic compounds. A method was developed for simultaneous separation, identification and quantification of both PAFFS and FA. Quantification was performed by the internal standard method using a five point regression graph of the UV-visible absorption data collected at the wavelength of maximum absorbance for each analyte. A total of sixteen phenolic compounds were tentatively identified and quantified. The content varied from 1.68 mg kg^{-1} to 1547 mg kg^{-1}. Results from HPLC analysis of the samples showed that gallic acid, (+)catechin hydrate and (-) epicatechin gallate were the major phenolic compounds present in the sample. Malvidin and pelargonidin 3-O-glucoside were the major anthocyanins.

Introduction

In the past few years there has been a concerted interest in studying and quantifying the phenolic compounds of red fruits due to their health-promoting properties. Grape phenolics include a wide range of compounds with antioxidant activity, such as phenolic acids, flavanols, flavonols, stilbenes, and anthocyanins. The phenolic composition of fruits varies greatly among cultivars. The concentration and composition of phenolics in red wine grapes vary with species, variety, season, and a wide range of environmental and management factors such as climate, soil conditions, canopy management, and crop load (Jackson and Lombard 1993). The extraction of phenolic compounds is primarily influenced by their sample particle size, the extraction method, and storage time (Maier 2008). Polar solvents, such as methanol, acetone, and acetonitrile yield high extraction efficiencies. However, they also extract other undesirable polar compounds present in samples (Alonso 1998). Satisfactory recoveries of phenols from soils and sediments have been reported with this technique using, in most cases, mixtures of polar and apolar organic solvents (Rodríguez-Montealegre 2005). Reverse-phase HPLC using photodiode array detection has been extensively reported for the identification and quantification of phenolic compounds in grapes and wine (Gao 1995b). Nevertheless, most of these methods target only certain classes of phenolic compounds (example: flavonoids and stilbenes vs. anthocyanins) whereas, simultaneous determination of all compounds is fairly unusual (Lin 2007).

Materials and Methods

Solvents and Reagents. Methanol, acetonitrile, acetone, petroleum ether, and phosphoric acid were purchased from Fisher Scientific (Fair Lawn, NJ) and were of analytical or HPLC grade. Water was from Milli-Q purification system Millipore (Millipore, Bedford, MA, USA). Ascorbic acid, ethyl acetate and β- glucuronidase type HP-2 from *Helix pomatia* were purchased from Sigma Aldrich (St Louis, MO, USA. Gallic acid, Ferulic acid, Caffeic acid, p-coumaric, (+)Catechin hydrate, Quercetin, (-) Epicatechin gallate, Isorhamnetin, Myricetin, trans-resveratrol, 7-ethoxycoumarin and β-glucosidase were purchased from Fluka (St Louis, MO. USA). The 3-*O*- glucosides of delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin were obtained from Polyphenols Laboratories AS (Sandnes, Norway). Sep-Pak C_{18} cartridges (1g, 6 mL) were obtained from Waters Corporation (WAT051910, Waters Corp., Milford, MA, USA).

Grape samples. Red commercial dried grape pomace was obtained from Vinifera for life (Canada).

Quantitative extraction protocols

Quantitative extraction I (phenolic acids, flavonols, and stilbenes). This extraction was modified from Torres (Torres 2005a). Juice was first pre-treated with HCl in order to hydrolyze sugar and final concentration of HCl in juice was 0.1%. Treated juice was centrifuged for 3,000 x g for 15 min prior to sampling.

Briefly, 0.5 g of sample was weighed (A-160, Denver Instruments Co) and transferred to a 30 mL brown bottle (Glass Amber with Teflon face lined cap, Fisherbrand, ThermoFisher Scientific Inc.) and 25 µL of 25 ppm 7-ethoxycoumarin (internal standard) and 4 mL of 50% v/v

methanol-water mixture were added. The bottle was placed in an ice bath and the mixture was stirred for 1 h. The mixture was subsequently centrifuged (Clinical 50-82013-800 centrifuge VWR International, Chicago) at 3,000 x g for 20 min and decanted through whatman filter paper (#41) into a 10 mL volumetric flask. Samples were then re-extracted under the same conditions and the combined filtrates were brought to volume with 10 mL of 50% methanol-water mixture. An aliquot of 2 mL was placed into a brown vial (3 mL Glass Amber with Teflon face lined cap, Fisherbrand, ThermoFisher Scientific Inc), to which 110 μL of 0.78M acetate buffer (pH 4.8), 100 μL of ascorbic acid and 50 μL of β-glucuronidase were added. Vials were capped, vortexed and incubated at 37°C for 17 h (overnight). Samples were centrifuged at 4,000 x g for 25 min and analyzed by RP- HPLC (Thimothe 2007b).

Quantitative extraction II (flavonoid-anthocyanins). The extraction was adapted from Kammerer (Kammerer 2004b) with some modifications. Briefly, 5 grams of sample, 200 μL of the internal standard 25 ppm 7-ethoxycoumarin and 100 mL of methanol/0.1% HCl (v/v) were combined and mixed in a brown bottle for 1 h under stirring and flushing with nitrogen in order to prevent oxidation during extraction at room temperature. The extract was centrifuged at 4,000 x g for 10 min, and the material was re-extracted with 100 mL of the organic solvent under the same conditions for 15 min. A 5 mL aliquot of the combined supernatants were evaporated to dryness under nitrogen water bath (Zymark TurboVap, Zymark Center, Hopkinton, MA) at 30°C to remove the organic solvent and the residue was dissolved with 2 mL of acidified water (pH 3.0, acetic acid). Anthocyanins were analyzed by RP-HPLC (Thimothe 2007b).

Preparation of crude extracts. The extraction conditions (time, solvent to solid ratio, temperature) were based upon literature data (Ju 2003) and previous extraction experiences of the research group (Vassan 2009). Conditions during all crude extraction experiments were:

solvent-to-ratio of 40 mL solvent per 20 gram extraction material and extraction time 1 h. The solvents were removed by nitrogen evaporation at 35°C. The extraction solvents used were: 70% methanol-water, 50% acetone-water, 0.01% pectinase- water mixture and petroleum ether.

Crude extraction of the polyphenols from pomace powder was conducted by weighing 20 grams of sample into 125 mL erlenmeyer flasks. The appropriate solvent, 40 mL, was added. The flasks were placed in a shaker (Classic C76, New Brunswick Scientific, Edison, NJ) maintained at 18°C and 250 rpm for 1 h. After shaking, samples were filtered under vacuum using a Buchner funnel with 5.5 cm diameter (55mm #1, Whatman Inc. Ltd., Mainstone, England). Samples were filtered until no visible dripping and then rinsed twice with approximately 10 mL of solvent for two subsequent filtrations. The final filtrates (except petroleum ether extracts) were transferred to 100 mL volumetric flask and brought up to volume with the corresponding solvent. The petroleum ether extracts were allowed to evaporate and were re-suspended in 100% acetone. A 10 mL aliquot was subsequently evaporated to dryness with nitrogen in a water bath at 30°C, dissolved in 7 mL of Milli-Q water, and applied to solid phase extraction cartridges (WAT051910, Waters Corp., Milford, MA). Aliquots of 5 mL were applied to the cartridges, which were activated with 5 mL of methanol, rinsed with 5 mL of deionized water and 3 mL of 0.01% HCl (v/v). Samples were eluted with 5ml of methanol and filtered through 0.45μm nylon filters (Fisherbrand, PTFE, Fisher Scientific, Denver, CO) and used for RP-HPLC analysis (Thimothe 2007b).

RP-HPLC analysis. The RP-HPLC procedure utilized was modified from Timothe (Thimothe 2007b). The method was designed to separate 17 phenolic compounds. Phenolic acids, flavanols, flavonols, stilbenes (PAFFS), standards were received as individual compounds. Flavanoid-anthocyanin (FA) standards were received as a mixture. A 100 ppm standard solution

containing all (11) of the individual PAFFS, in addition to the FA mixture (6) was prepared. This standard solution also contained 25 ppm internal standard (7-ethoxycoumarin). The standard curve was prepared by serially diluting (1:1) to a final concentration of 0.78 ppm. Separation, identification and quantification of individual phenolic compounds were performed on a reversed phase chromatography system (Alliance Waters 2690, Waters, Ireland) with a photodiode array detector (PDA, Waters 2996) and Empower 2 software (waters). Compounds were separated by a gradient elution system on a Sun Fire™ C18 column (5µm particle size, 4.6 x 250 mm i.d.) including a guard column (5µm particle size, 4.6 x 30 mm) at 25°C. The flow rate was set to 1.0 mL/min. For gradient elution, mobile phases A and B were employed. Solution A contained 0.1 % H_3PO_4 in MilliQ water, and solution B contained 0.1 % H_3PO_4 in acetonitrile (HPLC grade). Data acquisition was applied for 45 min with a total run of 65 min. Gradient elution was as follows: 92% A/8% B, at 0 min; 85% A/15% B at 5 min; 40% A/60% B at 45 min; 40% A/60% B at 55 min; and back to initial conditions 92% A/8% B at 60 min. The PDA was set at 210-600 nm and chromatograms were extracted at 280, 320, 370 for phenolic acids and flavonoids, and 520 nm for anthocyanins.

Statistical analysis. Data were analyzed using ANOVA to determine differences among solvent means using PROC GLM of Statistical Analysis SAS 9.2 (Cary, NC) version 9.2. (SAS Inst. 2003). Multiple comparison among the five organic solvents (50% Methanol, 70% Methanol, 50% Acetone, 0.01% Pectinase and petroleum ether) were analyzed each with two analytical replicates. Means were separated by Tukey's ($P < 0.05$). All experiments were conducted in triplicate.

Results and discussion

RP-HPLC analysis. Several factors such as maximum absorbance, retention time, mobile phases and concentration were studied to develop a method capable of resolving a large number of the phenolic compounds that are present in grape. This method differed from Timothe (Thimothe 2007b) by increasing the amount of the organic mobile phase (B) from 11% to 15% at 5 min and maintaining that amount of the organic mobile phase in isocratic elution for 40 minutes. This change allowed all the phenolic compounds investigated in this study to be eluted using a single HPLC method.

Quantitative extraction I (phenolic acids, stilbenes, and flavonols). In order to evaluate the effectiveness of the crude extractions of pomace, data was collected from a "quantitative" extraction. Quantitative extraction of phenolic compounds from grape has been previously reported (Kammerer 2004b; Thimothe 2007a). The extractions typically include the utilization of enzymatic hydrolysis to simplify chromatographic data. The Enzyme β-glucuronidase from *H. pomatia* Type-HP-2 is used to cleave the sugar moiety off of phenolic glycosides (Yáñez 2007). In addition, it was reported that β-glucuronidase contained arylsulfatase activity and can also effectively deconjugate flavonoid glucosides in red fruits (Thompson 2008).

The levels of individual and total PAFFS (phenolic acid, flavanols, flavonols and stilbenes) and FA (flavonoid-anthocyanids) measured in pomace are displayed in **Table 1**. The PAFFS concentration of the analytes measured in pomace was 1992 mg/kg dry matter, which is in agreement with the results found by Mazza (Mazza 1999), who reported total phenolic composition of 1000.9 mg of gallic acid dry matter in Cynthiana grape pomace extract.

Quantitative extraction II (flavonoid- anthocyanins). In the past, several studies have classified more than fifteen anthocyanidin glycones (Harborne and Williams 2000). In this study only six of them were identified and corresponded to cyanidin 3-*O*-glucoside (Cy3G), delphinidin 3-*O*-glucoside (Dp3G), malvidin 3-*O*-glucoside (Mv3G), pelargonidin 3-*O*-glucoside (Pg3G), peonidin 3-*O*-glucoside (Pe3G), and petunidin 3-*O*-glucoside (Pt3G) present in red fruits. Acidic solvents can cause the hydrolysis of glucoside groups joined to flavonoids, thus enhancing their migration into the solvent (Mazza 1995). As can be noticed, the recovery of FA was lower than for PAFFS. Nagel found that total anthocyanins were lower than total phenolics in Cabernet with values of 0.86 C3GE (Cyanidin 3-glucoside equivalent) mg/g and 3.69 GAE (Gallic acid equivalent) mg/g respectively (Nagel 1979)

Crude extracts. Extraction of phenolic compounds from commercial dried pomace using acetone: water, methanol: water and water has been previously reported (Revilla 2003; Lapornik et al. 2005; Ju 2003). The effectiveness of crude extractions in recovering PAFFS and FA from pomace was measured by comparing results to the previously described quantitative extractions. Significant differences were found among the solvents used for extraction of phenolic compounds in commercial dried pomace ($P < 0.05$) (**Table 2**). The concentration of FA using 50% methanol showed the highest recovery 26 mg/kg dry matter. However, this solvent was not the most efficient recovering PAFFS. The highest concentration of PAFFS among the solvents was obtained using 50% acetone with recoveries of 196 mg/kg dry matter.

Comparison of solvent recovery of phenolic compounds on commercial pomace.

Both the Quantitative extraction I (QI) and Quantitative extraction II (QII) of pomace performed strongly in the recovery of phenolic compounds. The quantitative protocols were

superior in recovering phenolic compounds from pomace than crude extracts. The recovery of PAFFS and FA from pomace (**Table 2)**, 50% methanol and 50% acetone were the most effective solvents (*P* <0.05). The PAFFS values of extracts when using 50% methanol solvent was the highest among the other solvents (1992 mg/kg). However, the FA values of extracts when using 70% methanol was the highest among the other solvents (26 mg/kg). Other researchers have reported that grape pomace not only has a high content of FA compared to the other samples, but also appears to have a higher content of unknown compounds (Thimothe 2007b). Similar observations were made in the current study (**Figure 3**). These unknown peaks were also detected on whole grape sample and can be related to acylated anthocyanins, which are present in abundance in grapes (Hong 1990).

Conclusions

In the present study, identification and quantification of the major phenolic acids, flavonoids and anthocyanins present in commercial dried grape pomace were established by chromatographic profiles. The results confirm that commercial dried grape pomace and its by-products are potentially sources of natural phenolic compounds. The type of solvent used for extraction affected quantity and composition of phenolic compounds in pomace extracts. It was generally observed for that by using quantitative extraction I, which yields the highest recoveries of phenolic compounds.

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Table 1. Content of phenolic compounds identified in commercial dried pomace (mg/kg ± RSD) when extracted quantitatively. Results are reported on a dry matter basis. PAFFS = phenolic acids, flavonols, flavanols and stilbenes. FA = Flavonoid-anthocyanins. Data are the mean for three replications.

	Analyte	50% Acetone	70 % Methanol	0.01% Pectinase	Petroleum Ether	Quantitative extraction I
PAFFS	(-) Epicatechin gallate	32.98 ± 0.62	9.87 ± 0.19	2.69 ± 1.23	12.89 ± 0.40	58.98 ± 0.09
	(+) Catechin hydrate	35.45 ± 0.55	18.74 ± 0.35	16.76 ± 0.85	1.83 ± 0.41	1278.28 ± 0.19
	Caffeic acid	< 0.1	< 0.1	0.34 ± 0.25	< 0.1	5.83 ± 0.54
	Ferulic acid	2.67 ± 1.08	< 0.1	1.75 ± 0.85	9.18 ± 0.28	9.17 ± 0.04
	Gallic Acid	10.31 ± 0.25	12.09 ± 0.34	26.86 ± 0.39	< 0.1	502.32 ± 0.036
	Isoharmnetin	13.21 ± 1.06	18.52 ± 0.51	< 0.1	1.66 ±0.16	21.74 ± 0.47
	Kaempferol	1.76 ± 1.02	2.11 ± 0.63	0.49 ±0.01	0.80 ± 0.26	24.60 ± 0.24
	Myricetin	1.92 ± 1.02	0.86 ± 0.65	0.24 ± 0.05	0.56 ± 0.14	16.48 ± 0.24
	p-coumaric acid	8.49 ± 0.86	3.69 ± 0.59	0.91 ± 0.47	2.76 ± 0.40	24.85 ± 0.37
	Quercetin	90.18 ± 1.16	14.58 ± 0.74	< 0.1	10.89 ± 0.27	50.00 ± 0.65
	Resveratrol	0.85 ± 0.16	< 0.1	< 0.1	< 0.1	< 0.1
	TOTALS	196.97 ± 8.37[b]	80.39 ± 2.25[b]	47.04 ± 5.33[b]	40.58 ± 1.78[b]	1992.25 ± 128.99[a]
FA	Cy3G	1.18 ± 0.01	4.58 ± 0.82	0.39 ± 0.02	< 0.1	3.63 ± 0.58
	Dp3G	< 0.1	2.38 ± 0.81	< 0.1	< 0.1	9.29 ± 0.60
	Mv3G	2.25 ± 1.64	13.93 ± 1.17	< 0.1	< 0.1	5.49 ± 0.74
	Pe3G	2.23 ± 0.37	< 0.1	< 0.1	< 0.1	1.44 ± 0.23
	Pg3G	0.61 ± 0.40	3.21 ± 0.49	0.23 ± 0.05	0.19 ± 0.04	4.12 ± 0.55
	Pt3G	< 0.1	2.48 ± 0.67	< 0.1	< 0.1	< 0.1
	TOTALS	6.28±028 [b]	26.60± 0.97[a]	0.62 ± 0.08[c]	0.19 ± 0.04[c]	23.98± 1.30[a]

[a-c] Means with similar letter are not significantly different (Tukey, $P > 0.05$)

CHAPTER V

UV-VIS SPECTROSCOPYANALYSIS OF PHENOLIC COMPOUNDS FRACTIONATED FROM GRAPE POMACE (*VITIS AESTIVALIS*) USING A POLYMERIC ADSORPTION VERSUS CELLULOSE ION EXCHANGE RESINS

Abstract

The separation of phenolic compounds (non-flavonoids and flavonoids) in grape pomace (*Vitis aestivalis*) by either adsorption (XAD) or ion exchange (IEX) resin was studied. Grape pomace was extracted with either a 50% solution of methanol or with a 50% solution of acetone for 1 h at room temperature. Extracts were applied to column resin and eluted with a mixture of $CH_3CN/TFA/H_2O$ (50:0.5:49.5 v/v/v). Phenolic compounds were obtained by collecting 5 mL fractions. These were evaporated to dryness under nitrogen and dissolved in MilliQ water. The intense red colored fractions were subjected to pH measurements and scanned with a spectrophotometer. A dilution 1:1 was used to correct the original absorbance. Characterization of the fractions by ultraviolet (UV)/visible (vis) spectroscopy confirmed the presence of phenolic compounds at 280, 320, 370 and 520 nm. The highest absorbance means using acetone extraction on IEX (ion exchange) resin at 280, 320 and 370 were 3.20, 3.20 and 2.86 absorbance units (AU) were obtained. The highest absorbance means using acetone on XAD resin

(Amberlite XAD 7 HP) at 280, 320 and 370 were 1.16, 0.66, and 0.26. At 520 nm absorbance readings were 0.08 AU for XAD and 1.28 AU for IEX. For methanol extracts absorbance means varied from 1.66 to 2.82 AU for XAD and for IEX ranged from 1.68 to 3.18 AU. The maximum absorbance means at 520 nm with methanol extracts were 1.66 AU and 1.48 AU for XAD and IEX resins, respectively. Fractionation of phenolic compounds in grape pomace was significantly ($P < 0.05$) affected by solvent and resin type. Furthermore, resin adsorption might also be a useful tool not only to concentrate plant phenolic compounds but also to fractionate the crude extracts or at least to enrich certain compounds.

Introduction

Although grape pomace is one of the most attractive residual sources of valuable phenolic compounds, it is mainly just used as a soil conditioner. The phenolic compounds in red grapes comprise simple phenolic acids (e.g. hydroxybenzoic acid, hydroxycinnamic acids and stilbenes) and complicated flavonoids (e.g. flavonols, flavanols, anthocyanin and tannins), which are extracted from grape skins and seeds during the vinification process (Macheix 1990b). Phenolic compounds isolated from plants have a diversity of high molecular structures and perform a wide range of different functions (Bravo 1998). Those compounds exhibiting one or more phenolic OH groups, have antioxidant activity and maintain the reduction reactions in plant (Kraemer-Schafhalter 1998). They also can be utilized as natural colorants or coloring agents (Bonilla 1999a). A major obstacle in the analysis of phenolic compounds derived from plants is their high content of sugar, which complicates organic solvent extractions utilizing ether, acetone or methanol (Zhang 2009). This problem is critical when the amount of the phenolic compounds in the material is small. As a result, numerous approaches for recovery of phenolic compounds

from plant processing by-products and their purification and fractionation have been proposed in recent years (Kammerer 2010; Maier 2008; Schieber 2003). Such fractionation steps may enhance antioxidant properties of phenolic compounds mixtures by reducing antagonistic effects (Iacopini 2008). Furthermore, extract components, such as peptides, proteins and sugars, may also interact with the phenolic compounds, thus affecting their antioxidant activity or causing color and flavor changes (Synge 1975; Zagorodni 2007).

The use of non-ionic, macro-reticular aliphatic cross-linked polymeric resins (Amberlite XAD) has increased the recovery of phenolic compounds from plant extracts by reducing soluble contaminants (Yang 2008). The use of adsorbent resins has enabled the potential isolation and recovery of compounds from plant sources as natural health promoting food supplements. Adsorption enables the separation of selected compounds from dilute solutions (Rosler 1984). The technique is attractive for its relative simplicity of design and high capacity rate. Additionally, it avoids using toxic solvents and minimizes degradation (Oprea 2006). Polymeric resins are made in 3-D networks by cross-linking hydrocarbon chains. The resulting resin is insoluble, inert and relatively rigid. Ionic functional groups are attached to this framework. Amberlite XAD 7 HP is a moderately polar XAD resin (Rohm 1969). It has been used to remove polar compounds from non-aqueous solvents, and to remove non-aromatic compounds from polar solvents. It has been used for removal of organic pollutants from aqueous wastes, ground water and vapor streams (Holloway 1973).

Ion exchange (IEX) resins are composed of a polymer matrix composed of inorganic compounds, polysaccharides, or synthetic resins, and a functional group (Levison 1997). Depending on the positive or negative charge of the ion active group, the resins act as cation or anion exchangers and depending on the affinity for counter ions each type can act as strong or

weak exchangers (Bonorden 1986). The strong cation exchangers contain sulfonic acid functional groups, while weak cation exchange resins contain carboxylic acid functional groups. Both are based on spherical particles manufactured from cross-linked cellulose containing carboxyl methyl groups. Some of the advantages of using ion exchange resin is pure phenols and natural extracts are absorbed, purified and analyzed in less time (Ku 2000; Raman 2005).

Absorbance measurements are simple techniques to measure the color of grape extracts and wine. Spectroscopic analysis has been used to study the effect of grape pomace on phenolic composition. Various classes of phenolic compounds are detected and characterized by ultraviolet (UV)-Visible (vis) in Cynthiana grape pomace (Rosler 1984). The aim of the present study was to compare the isolation of phenolic compounds on Amberlite XAD 7 HP and cellulose ion exchange from grape pomace using acetone and methanol-water mixture as polar solvents by UV-Visible spectroscopy.

Materials and Methods

Solvents and Reagents. All reagents were purchased from Fisher and were HPLC grade. Water was from Milli-Q purification system Millipore (Millipore, Bedford, MA, USA) was used throughout. Amberlite® XAD 7 HP Industrial Grade Polymeric Adsorbent was purchased from Room and Haas Corp. Philadelphia, PA and C M C-500 ion exchange chromatography media was obtained from Amicon Matrex, Cellufine Corporation, Danvers, Mass.

Pomace preparation. Grape pomace from Cynthiana (*Vitis aestivalis*) was prepared using a small pilot scale winemaking process. Approximately 30 pounds of frozen grapes were weighed and thawed at 4ºC for two days. Grapes were destemmed and gently crushed with a

commercial grape destemmer-crusher (Jolly-60, St. Patrick's of Texas, Austin, TX). They were placed into a 100 liter (25 gallon) stainless steel fermentation vessel for maceration and wine grade yeast (*Saccharomyces cerevisiae*) and yeast nutrient (Fermaid) was also added. The vessel was capped with adjustable height lids allowing approximately 25cm (≈10 inches) of headspace at 20-22°C for 5 days in order to mimic industrial process. Samples were monitored by rapid residual sugar tests (AV-RS Accuvin LLC, Napa, CA). The lid was pressed down to minimize head space 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 wine and pomace (Jensen 2008a). Samples of pomace were collected in vacuum bags (16 x 25 inch vacuum bags, Curwood, Inc, Oshkosh, WI) and stored at -20°C until further analysis. Wine samples were discarded. After pressing, pomace was frozen using a metal strainer and liquid nitrogen (-196°C). Sample was ground for 30s in a 4°C room using a Waring blender container (model 51BL31) previously stored in a freezer. The resulting powder was stored in vacuum bags (8 x 10 inch vacuum pouches, Mid-Western Research & Supply, Inc) at -20°C until further analysis.

Extracts preparation. Grape pomace was extracted with either 50% methanol or 50% acetone. Briefly, twenty grams of ground grape pomace and 40 mL of solvent were combined and agitated using a shaker (Classic C76, New Brunswick Scientific, Edison, NJ) maintained at 18°C and 250 rpm for 1 h. After extraction the extracts were filtered using a Buchner funnel (5.5 cm diameter) on a 250 mL vacuum flask through Whatman filter paper (55mm #1, Whatman Inc. Ltd., Mainstone, England).

Column Preparation

Adsorptive resin. Approximately 100 g of Amberlite® XAD 7 HP (XAD) resin was slurried in 300 mL of MilliQ water for 1 h before use. An econo-column (2.5 X 30 cm, glass chromatography column, max vol. 147 mL, cross-sectional area 4.91 cm^2, for use in low pressure chromatography Bio-Rad Laboratories, Inc., Hercules, CA) was washed with 25 mL of Milli-Q water and filled with the slurry of the polymeric resin, using a clean beaker. Occasionally, the excess of water was drained through the bottom of the column to prevent overflow. The 100g of slurried resin was poured into the column. The material was subsequently rinsed first with 150 mL of MilliQ water followed by 50 mL acidified water (triofluoracetic acid, pH 2.0) at a flow rate of 1.5 mL/min using a econo-column® Flow adaptor (2.5 cm column ID, 1-14 cm functional length, Bio-Rad Laboratories, Richmond, CA). Sufficient solvent was added to submerge the adsorbent. A backwashing procedure was designed in order to release the entrapped air by attaching a water line to the bottom of the column and so water could flow-up the column. This flow was maintained until all air pockets were removed and all the particles had achieved mobility (usually to 5-10 min). This step was important to obtain a good particle size segregation with the smaller particles at the top and the larger ones at the bottom portion of the column.

Cellulose Ion exchange. An econo-column was washed with 25 mL of MilliQ water and filled with 40 mL of ready to use cellulose ion exchange (IEX) resin. The column was washed two times with 50 mL of MilliQ water.

Fractionation of extracts. Extracts were fractionated as described by Nørbæk and Kondo (Nørbæk 1999) with some modifications. Extracts were poured into a resin containing column. The eluate was discarded and the resin was subsequently washed with a 100 mL

mixture of $CH_3CN/TFA/H_2O$ (50:0.5:49.5 v/v/v). Phenolic compounds were eluted using CH_3CN/TFA (99.5:0.5 v/v) and thirty five 5 mL fractions were collected from the eluate. These were evaporated to dryness under nitrogen water bath (Zymark TurboVap, Zymark Center, Hopkinton, MA) at 30°C to remove the organic solvent and the residues were dissolved in 2 mL of MilliQ purified water and pH was measured using accumet basic pH meter (AB15 plus pH meter, Fisher scientific). All experiments were performed in duplicate.

Ultraviolet–visible spectroscopy. The UV–visible spectra of the diluted data (1:1) of the pigmented red fractions eluted from XAD and IEX resins were recorded on an Ultraviolet/Visible Beckam counter DU 7500 spectrometer from 200 to 700 nm (Beckam instruments, Inc., Fullerton, CA). The fraction read/plot and scanning mode was used to analyze samples (diluted fractions). Maximum absorbance of fractions was also measured at 280, 320, 370 and 520 nm. Readings were in triplicate.

Statistical Analysis. Significant differences between resins, solvents and wavelengths were determined using Tukey test ($\alpha = 0.05$) for the comparison of extraction solvent. The *t*-test ($\alpha = 0.05$) was used for paired random samples, to verify significant differences of phenolic compounds content.

Results and discussion

Fractionation of the extracts. Significant differences were found among the fractions eluted on resins and solvents ($P < 0.05$). The fractionation of phenolic compounds with XAD and IEX resins using methanol:water and acetone:water was studied. All fractions were screened in read/plot scanning mode in order to select the ones with higher absorbance. After the screen, the red intense fractions resulted to be the best regarding to absorbance. For methanol:water extracts, red pigmented eluates were obtained from the first 28 fractions (140 mL) collected from

the XAD resin. Fraction pH values ranged from 6.4 to 7.0. In addition, the IEX resin also resulted in red pigmented eluates in the first 35 fractions (175 mL) and pH values ranged from 6.1 to 6.5.

For acetone:water extracts, highly red pigmented eluates were obtained from the first twelve fractions (60 mL) using the XAD resin and pH values ranged from 6.0 to 6.4. The IEX resin red pigmented eluates were only collected in the first six fractions (30 mL) and pH values ranged from 5.9 to 6.3. The intense red color fractions obtained from the XAD and IEX columns were subjected to scan spectrometer in order to determine the presence of phenolic compounds at different wavelengths. Since the focus of this study was on the isolation of phenolic compounds from grape pomace, further quantification of active phenolic compounds was not taken into consideration.

Resin matrix dependent differences in phenolic compound recovery. A further purpose of the present study was to find differences in absorbance of phenolic compounds extracted with different solvents using adsorption and ion exchange resins at different wavelengths. **Table 1** reports the absorbance means of the corrected dilution factor (1:1) of the fractions at 280, 320, 370 and 520 nm using acetone:water or methanol:water mixture.

The absorbance means were calculated by measuring individual fraction at each wavelength. Measurements were averaged for each wavelength.

Absorbances at 280 nm. For the acetone:water mixture, the XAD resin absorbance mean from all red pigmented fractions was 1.16 AU higher and significant differences were found among wavelengths 280, 370 and 520 nm ($P < 0.05$) (**Table 1**). On the other hand, methanol:water mixture, the XAD resin absorbance mean from all red pigmented fractions was

2.68 AU higher compared to those found in acetone:water mixture. A significant differences were found between wavelengths 280 and 520 nm (P <0.05).

For the acetone:water mixture, the IEX resin absorbance mean from all red pigmented fractions was 3.18 AU higher and a significant difference was found between wavelengths at 280 and 520 nm found (P <0.05). In addition, for methanol:water mixture, the IEX resin absorbance mean from all red pigmented fractions was 3.18 AU higher and significant differences were found among wavelengths 280, 370 and 520 nm (P <0.05).

Absorbances at 320 nm. Red pigmented fractions collected from the XAD resin using acetone:water mixture had a mean absorbance of 0.66 AU, while those from the IEX resin had a mean absorbance of 3.2 AU. Significant differences in absorbance units were found between wavelengths 320 and 520 nm on XAD and IEX resins (P <0.05). Red pigmented fractions collected from the XAD resin using methanol:water mixture had a mean absorbance of 2.82 AU, while those from the IEX resin had a mean absorbance of 2.84 AU. Significant differences in absorbance units were found between wavelengths 320 and 520 nm on XAD and IEX resins (P <0.05).

Absorbances at 370 nm. Red pigmented fractions collected from the XAD resin using acetone:water mixture had a mean absorbance of 0.26 AU, while those from the IEX resin had a mean absorbance of 2.86 AU. Significant differences in absorbance units were found between wavelengths 370 and 520 nm on XAD and IEX resins (P <0.05). Red pigmented fractions collected from the XAD resin using methanol:water mixture had a mean absorbance of 2.52 AU, while those from the IEX resin had a mean absorbance of 1.68 AU. Significant differences in absorbance units were found between wavelengths 370 and 520 nm on XAD and IEX resins (P <0.05).

The results obtained in this study show the effectiveness of IEX resin for recovery of phenolic compounds and are in agreement with those found and explained by Lam (1970) and Tsutsuki (1984). They explained that because phenolic compounds are small molecules and more negatively charged than proteins, their absorbance decreases as the pH becomes more acidic causing a change in the structure associated with the dissociation of the –COOH groups and of the –OH groups of phenolic compounds. Also, the small particle diameter of the resin allows smaller phenolic compounds to pass through while retaining larger molecules. In addition, polar and hydrophobic binding also plays an important role. The polar interactions may include hydrogen bonding as well ion exchange (Olsson 1976).

Absorbances at 520 nm. In addition, the absorbance means for phenolic compounds using acetone:water mixture ranged from 1.48 AU using IEX to 1.66 AU using XAD. Our results confirmed previous studies by Lapornik (Lapornik 2005) which found the AU of anthocyanins were less than those measured at 280, 320, and 370 nm. This may be ascribed to the increase of the particle diameter and therefore to an enhanced wetting of the adsorption resin, which results in an enhanced solute transfer from the boundary fluid layer to the solid phase (Scordino 2003).

Ultraviolet–Visible Spectroscopy. UV–Vis PDA spectra suggested that several of the semi-purified aqueous fractions extracted with different solvents contained phenolic compounds using amberlite XAD 7 HP and IEX. The data was plotted as a function of fraction number and was found to be related to the differences in molecular weight of the fractions at different wavelengths.

Figure 1 shows the relative concentration of pigmented fractions eluted on XAD using methanol. As can be noticed, fractions 22 and 27 showed higher relative concentration at 280

nm with values of 3.7 and 3.4 respectively. Fraction 25 showed a higher relative concentration at 320 nm with 3.4 AU compared at 370 and 520 nm respectively. However, fractions 26 and 27 showed a higher relative concentration at 370 nm with 2.5 and 2.7 AU. At 520 nm, all the fractions showed a reduction in relative concentration, which ranged from 1.36 to 1.77 AU compared to 280, 320, and 370 nm.

Figure 2 shows the relative concentration of pigmented fractions eluted on XAD using acetone. Most of the red pigmented fractions showed higher relative concentration at 280 nm, except for fraction 8 with 0.8 AU compared to 320, 370 and 520 nm. However, an interesting trend was seen at 320 nm on fraction 9, which was very close to 280 nm with 1.15 AU. At 370 nm, fraction 9 showed a higher relative concentration with 0.88 AU compared to 320 and 520 nm but it was less than at 280 nm with 1.12 AU.

Figure 3 shows the relative concentration of pigmented fractions eluted on IEX using methanol. As can be noticed, most of the fractions showed a higher relative concentration at 280 nm except fraction 34 with 1.34 AU compared to 320, 370, and 520 nm. However, at 320 nm fractions 33 and 34 showed higher relative concentration with 3.2 and 3.4 AU compared to 370 and 520 nm. All the fractions showed less relative concentration at 370 from 0.8 to 1.0 AU and at 520 nm from 0.5 to 2.4 AU.

Figure 4 shows the relative concentration of pigmented fractions eluted on IEX using acetone. As can be noticed, most of the fractions at 280, 320 and 370 nm showed a higher relative concentration compared to 520 nm. At 280 nm the means of the relative concentration in the fractions ranged from 2.4 to 3.2 AU. At 320 nm the means of the relative concentration in the fractions ranged from 1.8 to 3.4 AU and at 370 nm the means of the relative concentration in the fractions ranged from 1.4 to 3.4. At 520 nm the means of the relative concentration in the

fractions ranged from 0.1 to 1.5 AU. It should also be noticed that the fractions collected on IEX using acetone absorbed more strongly in the UV-Vis than XAD at 280, 320 and 370 nm using acetone or methanol:water mixture.

Conclusion

Fractionation of phenolic compounds in grape pomace was shown to be significantly affected by solvent and resin type.

Therefore, the present study may contribute to the production of purified plant extracts with various health beneficial effects. Future studies will be need to characterize further resin materials in terms of their interaction with individual plant phenolic compounds. The data obtained in such studies can be used for the prediction and with it the optimization of purification and separation processes, which are based on the recovery of natural compounds using polymeric or IEX resins. Furthermore, resin adsorption might also be useful tool not only to concentrate plant phenolic compounds but also to fractionate the crude extracts or at least to enrich certain compounds.

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Table 2. Relative concentration means of red pigmented fractions using XAD 7 HP and IEX resin. Multiple comparisons were established by differences in AU at wavelengths per resin

	Amberlite XAD 7HP [x] λ nm				Ion exchange[y] λ nm			
Extraction	**280**	**320**	**370**	**520**	**280**	**320**	**370**	**520**
Acetone:Water[x]	1.16b	0.66b	0.26b	0.08b	3.2a	3.2a	2.86a	1.28b
Methanol:Water[y]	2.68a	2.82a	2.52a	1.66a	3.18a	2.84b	1.68b	1.48a

[a-d] Values within resin with similar letter are not significantly different (Tukey, $P > 0.5$)

[x,y] Values within treatments (resin or solvent) with similar letter are not significantly different (Tukey, $P>0.05$)

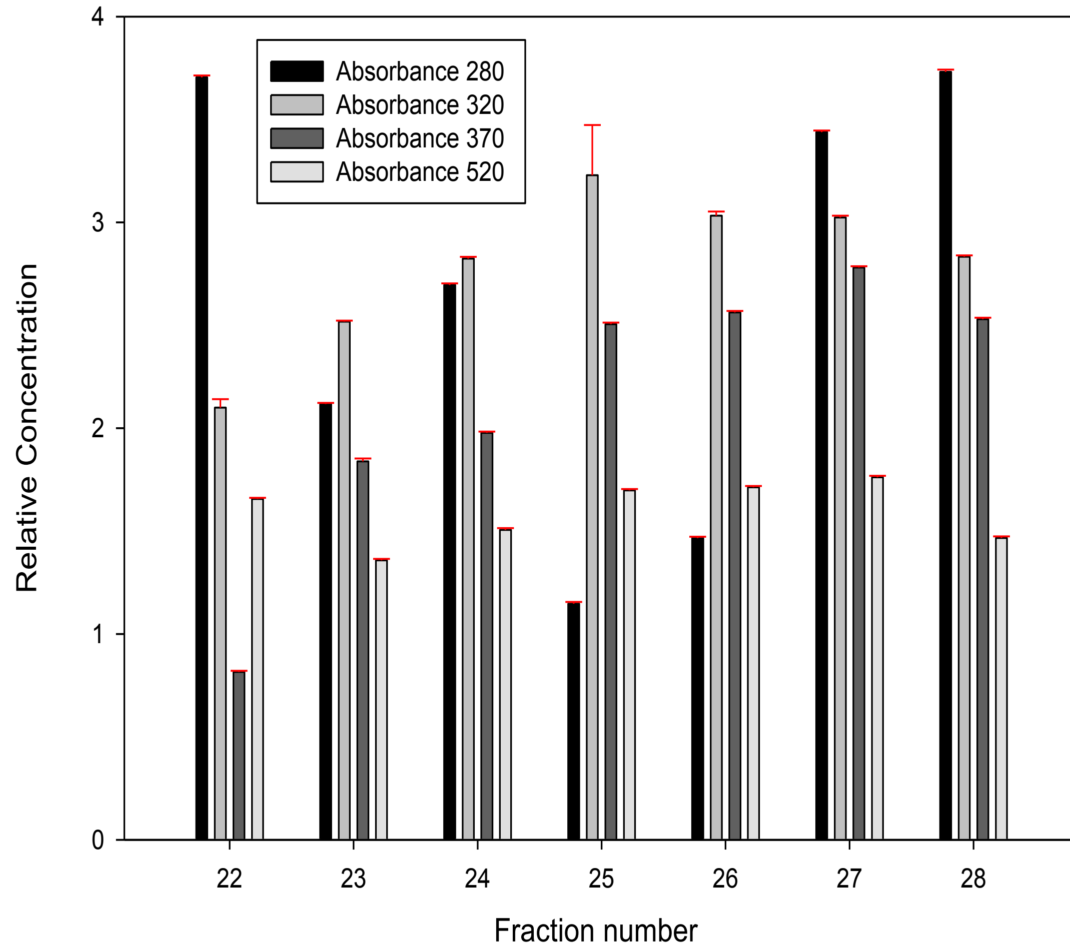

Figure 1. Relative concentration of red pigmented fractions eluted from Amberlite XAD 7 HP using methanol

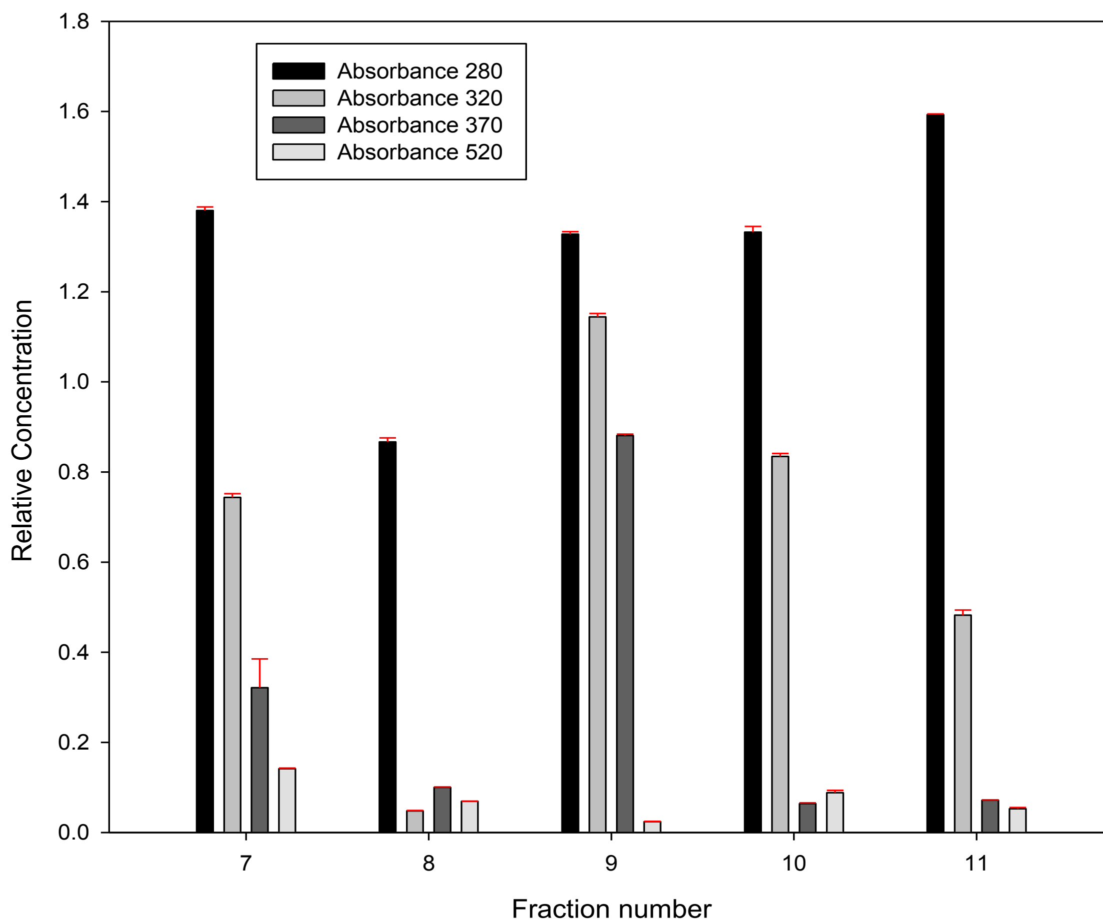

Figure 2. Relative concentration of red pigmented fractions eluted from amberlite XAD 7 HP using acetone

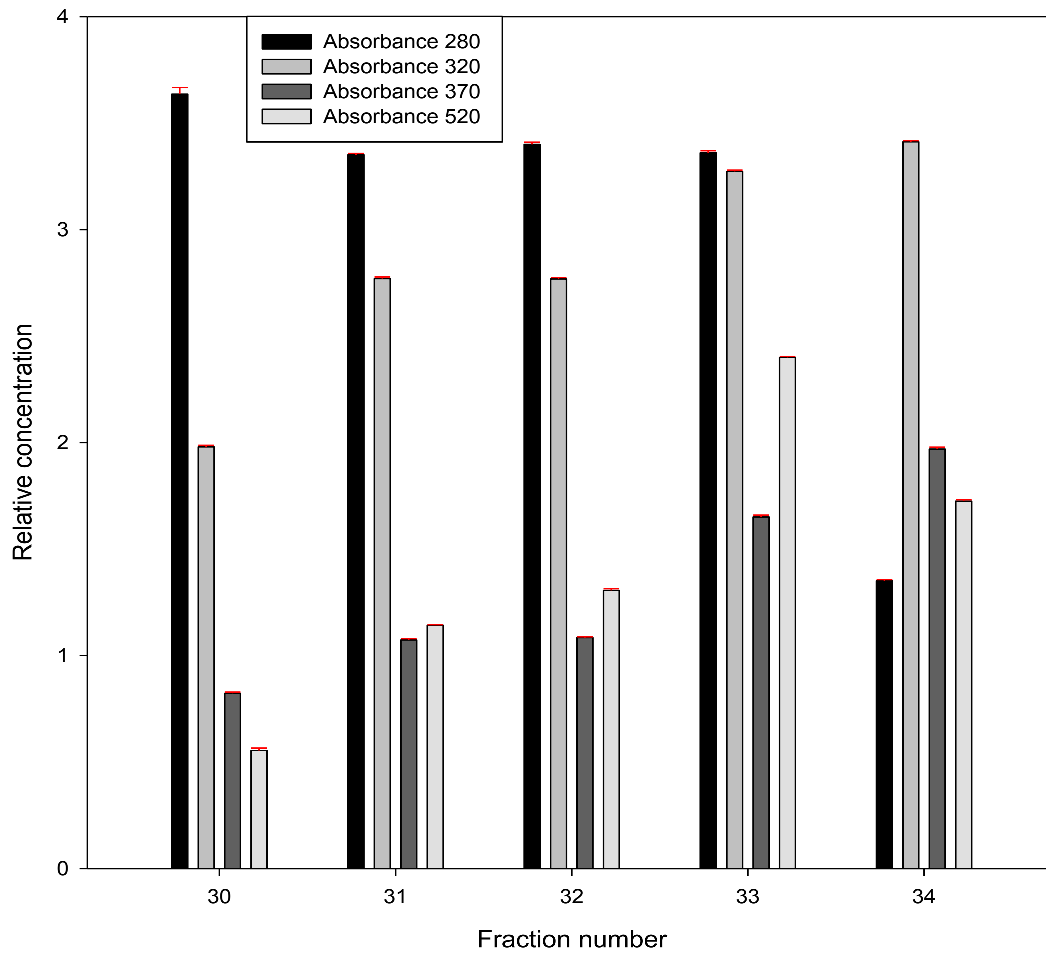

Figure 3. Relative concentration of red pigmented fractions eluted from IEX resin using methanol

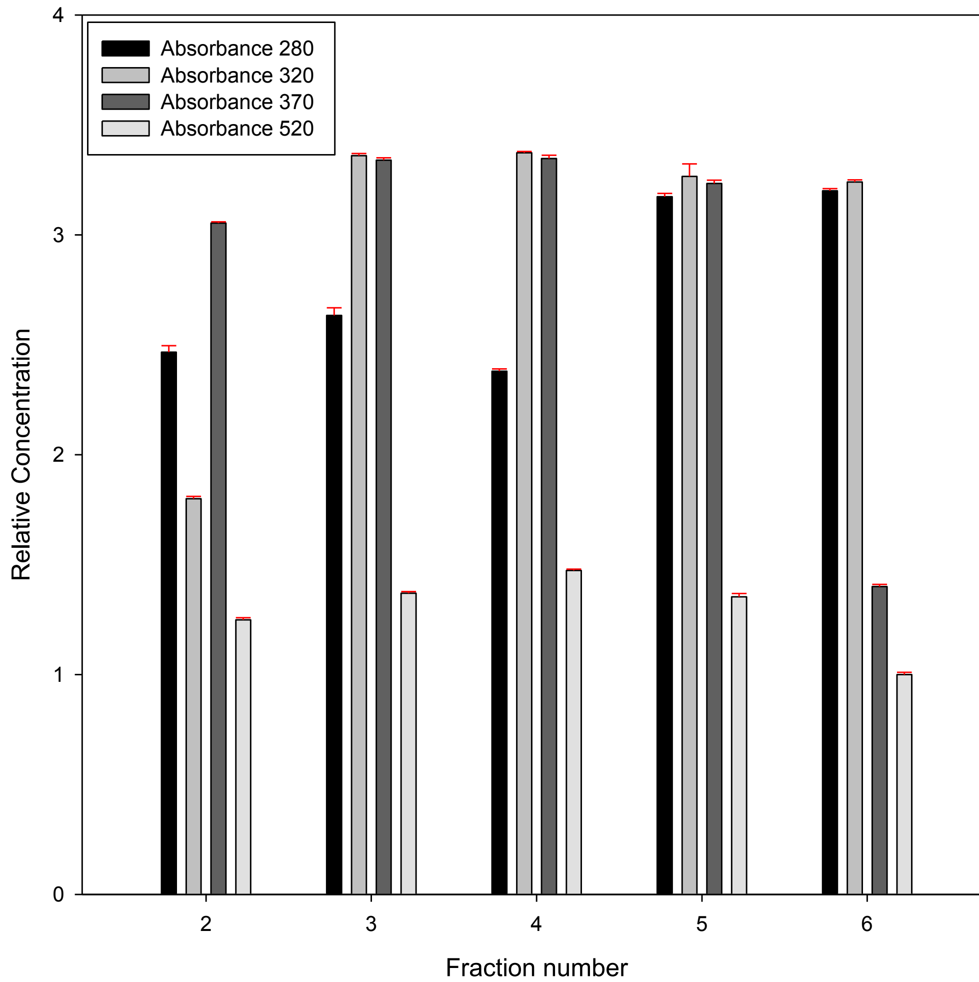

Figure 4. Relative concentration of red pigmented fractions eluted from cellulose IEX resin using acet

CHAPTER VI

ANTIBACTERIAL ACTIVITIES OF PHENOLIC COMPOUNDS ISOLATED FROM GRAPE POMACE (*VITIS AESTIVALIS*) ON *E.COLI 0157:H7, S. AUREUS* AND *L. MONOCYTOGENES*

Abstract

By-products obtained from winemaking processes still contain large amounts of phenolic compounds, especially phenolic acids, flavanols, flavonols, stillbenes and anthocyanins, which can? act as antimicrobials. Phenolic compounds from grape pomace were extracted using methanol:water or acetone:water. The extracts were fractionated and purified using Amberlite XAD 7HP (XAD) and cellulose ion exchange (IEX) resins to remove sugars. Aliquouts of 5 mL of the most pigmented fractions were evaporated to dryness and screened for antimicrobial activity against Gram-positive and Gram-negative bacteria using spot on the lawn assay. Data was measured as arbitrary activity units (aAU). Reversed phase high performance liquid chromatography (RP-HPLC with diode array detector was used to identify phenolic compounds. Quantification was performed by the external standard method using five point regression graph of the UV-visible absorption data collected at the wavelength of maximum absorbance for each analyte. The total phenolic content of the fractions eluted on XAD using acetone ranged from 40 to 476 mg/kg dry matter and using methanol varied from 26 to 39 mg/kg dry matter.

The total phenolic content of the fractions on IEX using acetone ranged from 2 to 13 mg/kg dry matter and using methanol ranged from 41 to 81 mg/kg dry matter. On comparing the antimicrobial activity of all the isolated fractions, it was found that XAD resin using acetone showed the highest units against all the tested bacteria. Results from spot on the lawn assay showed that bacterial strains exhibited diverse sensitivities towards different concentrations of phenolic compounds. The methanol fractions differed from acetone fractions by high content of anthocyanins.

Introduction

Phenolic compounds are cyclic benzene compounds with a minimum of one hydroxyl group associated directly with the ring structure. Based on their structure two groups are distinguished flavonoids and non-flavonoids phenols (Rodríguez Montealegre 2005a). Flavonoid phenols are subdivided into anthocyanins, flavanols, flavonols and tannins (Kennedy 2001; Allen 1998). Non-flavonoid phenols consist primarily of phenolic acid and esters (Singleton 1976). The differences between both groups are the number and orientation of phenolic-subunits with the molecules (Bowyer 2002a). Flavonoids are widely distributed in grapes, especially in seeds and stems, and principally contain anthocyanins. Anthocyanins are pigments and mainly exist in red grape skins. Phenolic acids in grapes comprise derivatives of hydroxycinnamic acid, including caffeic acid and p-coumaric acid (Rodríguez Montealegre 2005b; Kammerer 2004a). Recently, microbial-derived phenolic acids have been implicated in providing a variety of health benefits to the host, such as the inhibition of platelet aggregation (Rechner 2005; Aura 2008) and antiproliferative activity in prostatic and tumoral cells (Gao 2006). In addition, the antimicrobial activity of specific phenolic acids towards pathogens has been assessed while evaluating the antimicrobial properties of pure phenolics and plant extracts

(Si 2006). Most of the phenolic compounds found in plants have been demonstrated to have antimicrobial activities (Chávez 2006; Puupponen-Pimiä 2001; Rodriguez-Vaquero 2007). These activities have been studied in controlling invasion and growth of plant pathogens, their activity against food pathogens has been investigated to characterize and develop new healthy food ingredients, medical compounds, and pharmaceuticals (Puupponen-Pimiä 2005a; Cavanagh 2003). The mechanism of how phenolic compounds act is understood by the site and number of hydroxyl groups, which are related to the level of toxicity and are directly proportional to each other. Some researchers have described the mechanism of catechin and epicatechin to be substrate deprivation and membrane disruption respectively. Other mechanisms of action noted by the study of phenols in general and subclasses of phenolic acids, flavonoids and tannins involve enzyme inhibition, enzyme inactivation, formation of complexes with cell walls and metal ions (Cowan 1999). In addition, some authors have found that more highly oxidized phenols are more inhibitory through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason 1987). Other studies have showed the antimicrobial activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls as more lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya 1996a).

Many Gram-negative and Gram positive bacteria are difficult food contaminants and pathogens. Therefore, their control in food processing systems and food is vital. The difference between cells of Gram-negative and Gram-positive bacteria is that Gram-negative bacteria are surrounded by inner and outer lipidic membranes and Gram-positive bacteria do not have the inner membrane (Cowan 1999). As a result phenolic compounds specifically phenolic acids and

anthocyanins act as weak acids by donning H^+ causing a reduction in pH from neutral to acid in the bacterial cytoplasm producing toxicity and inhibition (Akiyama 2001; Scalbert 1991).

The objectives of this study were therefore to investigate the phenolic composition of grape pomace (*Vitis aestivalis)*, to isolate the phenolic compounds from this sample using XAD and IEX resin and to determine the antimicrobial activities of the isolates using spot on the lawn assay.

Materials and Methods

Chemicals. Methanol, acetonitrile, acetone, and phosphoric acid were purchased from Fisher Scientific (Fair Lawn, NJ and were of analytical or HPLC grade. Water was from Milli-Q purification system Millipore (Millipore, Bedford, MA, USA). Amberlite® XAD 7 HP Industrial Grade Polymeric Adsorbent was purchased from Room and Haas Corp. Philadelphia, PA and C M C-500 ion exchange chromatography media was obtained from Amicon Matrex, Cellufine Corporation, Danvers, Mass.

Pomace preparation. Grape pomace from Cynthiana (*Vitis aestivalis*) was prepared using a small laboratory scale winemaking process. Cynthiana pomace was produced on pilot-scale level by the following protocol: approximately 30 pounds of frozen grapes were weighed and thawed at 4ºC for two days. Grapes were destemmed and gently crushed on with a commercial grape destemmer-crusher (Jolly-60, St. Patrick's of Texas, Austin, TX). They were placed into a 100 liter (25 gallon) stainless steel fermentation vessel for maceration and wine grade yeast (*Saccharomyces cerevisiae*) and yeast nutrient (Fermaid) was also added. The vessel was capped with adjustable height lids allowing approximately 25cm (≈10 inches) of headspace at 20-22˚C for 5 days in order to mimic industrial process. Samples were monitored by rapid residual sugar tests (AV-RS Accuvin LLC, Napa, CA). The lid was pressed down daily

to minimize headspace 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 wine and pomace (Jensen 2008a). Samples of pomace were collected in vacuum bags (16 x 25 inch vacuum bags, Curwood, Inc, Oshkosh, WI) and stored at -20°C until further analysis. Wine samples were discarded. After pressing, pomace was frozen using a metal strainer and liquid nitrogen (-196°C). Sample was ground for 30s in a 4°C room using a Waring blender (model 51BL31) previously stored in a freezer. The resulting powder was stored in vacuum bags (8 x 10 inch vacuum pouches, Mid-Western Research & Supply, Inc) at -20˚C until further analysis.

Extraction of the phenolic fractions. Grape pomace was extracted with either 50% methanol or 50% acetone. Briefly, twenty grams of ground grape pomace and 40 mL of solvent were combined and agitated using shaker (Classic C76, New Brunswick Scientific, Edison, NJ) maintained at 18°C and 250 rpm for 1 h. After extraction the extracts were filtered using a Buchner funnel (5.5 cm diameter) on a 250 mL side arm Erlenmeyer flask through Whatman filter paper (55mm #1, Whatman Inc. Ltd., Mainstone, England).

Isolation and purification of grape pomace extracts. The extracts were futher purified by a method based on the work of Nørbæk and Kondo (Nørbæk 1999), using Amberlite XAD 7HP (XAD) and cellulose ion exchange (IEX) resins. The samples were introduced into the column (diameter 2.5 X 30 cm, glass chromatography column) and were eluted with a mixture of CH_3CN/TFA/H_2O (50:0.5:49.5 v/v/v). Finally, the columns were washed with CH_3CN/TFA (99.5:0.5 v/v) to elute the remaining phenolics. During sample application, elution of phenolic compounds was obtained by collecting fractions of 5 mL. These were evaporated to dryness under nitrogen water bath (Zymark TurboVap, Zymark Center, Hopkinton, MA) at 30°C to

remove the organic solvent and the residues were dissolved in 2 mL of MilliQ purified water for further HPLC analysis. All experiments were performed in duplicate.

HPLC analysis. The HPLC procedure utilized was modified from Timothe (Thimothe 2007a). The method was designed to separate several phenolic compounds including phenolic acids, flavanols, flavonols, stilbenes and anthocyanins. The standard curve was prepared by serially diluting (1:1) to a final concentration of 0.78 ppm. Separation, identification and quantification of individual phenolic compounds were performed on a reversed phase chromatography system (Alliance Waters 2690, Waters, Ireland) with a photodiode array detector (PDA, Waters 2996) and Empower 2 software (waters). Compounds were separated by a gradient elution system on a Sun Fire^{TM} C18 column (5µm particle size, 4.6 x 250 mm i.d.) including a guard column (5µm particle size, 4.6 x 30 mm) at 25°C. The flow rate was set to 1.0 mL/min. For gradient elution, mobile phases A and B were employed. Solution A contained 0.1 % H_3PO_4 in MilliQ water, and solution B contained 0.1 % H_3PO_4 in acetonitrile (HPLC grade). Data acquisition was applied for 45 min with a total run of 65 min. Gradient elution was as follows: 92% A/8% B, at 0 min; 85% A/15% B at 5 min; 40% A/60% B at 45 min; 40% A/60% B at 55 min; and back to initial conditions 92% A/8% B at 60 min. The PDA was set at 210-600 nm and chromatograms were extracted at 280, 320, 370 for phenolic acids and flavonoids, and 520 nm for anthocyanins. The phenolic compounds analyzed were: gallic acid, ferulic acid, caffeic acid, p-coumaric, (+) catechin hydrate, quercetin, (-) epicatechin gallate, isorhamnetin, myricetin, trans-resveratrol; anthocyanins 3-O-glucosides: cyaniding, delphinidin, peonidin, petunidin, pelargonidin and malvidin.

Bacterial strains and growth conditions. The bacterial strains used as test organisms were *Escherichia coli* ATCC 43888 , *Sthaphylococcus aureus* ATCC 51651 and *Listeria*

monocytogenes CW 77 (Gamble 2007). All bacteria were cultured aerobically at 37°C in nutrient broth and agar medium.

Before experimental use, cultures from solid medium were subcultured in liquid media, incubated for 24 h and used as the source of inoculums for each experiment.

Antibacterial test. The test used to investigate antimicrobial effects of phenolic compounds was spot on the lawn with a soft agar.

Inoculum preparation. Inoculum was prepared following the method of Lang (Lang 2004). Before each experiment, frozen stock cultures of *S. aureus, E. coli O157:H7* and *L. monocytogenes* CW 77 were streaked on tryptone soy agar (TSA, Difco, Becton Dickinson, Sparks, Md., U.S.A.). Cultures were incubated at 37°C for 24 h and an isolated colony of each strain of each pathogen was transferred to TSB (9 mL). At two consecutive 24-h intervals, further transfers were made in TSB using a sterile loop (approximately 10 µL).

Spot on the lawn assay. The spot-on-lawn assay (Carlson 2005; Hoover 1993; Kutter 2009) is an easy way to determine antimicrobial activity in samples. This technique involves first inoculating a bacterial culture on agar and then creating a pour plate. Small volumes of antibiotic containing diluent are subsequently added to a specific location (a spot). Positive bacterial killing, given sufficient antimicrobial is indicated by a clearing of the bacteria encompassing the original spot. The respective basal medium (TSA) was autoclaved and kept in a molten state in a water bath (Fisher Scientific, Isotemp 210, Fair Lawn, NJ) at 50°C for 1h. Aliquots of 1 mL of overnight culture with a cell density 10^8 -10^9 were added into 50 mL of molten medium. Approximately, 5 mL of inoculated medium were poured into a Petri dish (100x15mm, VWR International, Bridgeport, NJ). After cooling and solidification, the Petri dish was divided in eight equal sections, where sterilized paper discs (6 mm) were placed (6 BBL,

Becton, Dickinson and company, Sparks, MD). The individual fractions were serially 1:1 diluted 8 times in order to test the minimum inhibition concentration required to produce an inhibition zone. Then, 25 µL of each diluted fraction were added to the discs and plates were refrigerated at 4°C for 1h. Control agar plates were prepared similarly but without the grape pomace using same solvents as base. All Petri dishes were incubated at 37°C in the inverted position and observed after 48 h. Inhibition of the pathogenic bacteria by the spot-inoculated isolate was evident as a clear zone surrounding the growing spot culture.

Arbitrary activity unit assay. The reciprocal of the highest dilution (in a series of 1:1 dilutions) showing a zone of inhibition by spot-on-lawn technique was taken as the number of arbitrary units of inhibitor, and adjusted to obtain arbitrary units per ml (aAU/ml).

$$aAU/mL = \frac{reciprocal of the highest dilution \times 1000}{volume of the sample to be added}$$

Statistical analysis. Significant differences in phenolic composition among fractions eluted on resins were determined using the Tukey test (α = 0.05) for the comparison of independent samples. The relationships between antimicrobial units and content of total phenolic compounds in the fractions were determined using the Pearson correlation test. Data evaluation was performed using the SAS software package (SAS institute, Carry, NC, USA, Software version 9.2).

Results and discussion

HPLC analysis. From the red pigmented fractions, the isolated compounds were identified by HPLC according to the method previously described by Timothe (Thimothe 2007a). The majority of the isolated phenolics were identified by their UV spectra and their analytical HPLC retention times in comparison with standard compounds. The total phenolic

compounds measured in each fraction eluted on XAD and IEX using methanol and acetone are displayed in **Tables 1** and **2** respectively. The phenolic compounds found in collected fractions were identified as phenolic acids: gallic acid, caffeic acid, ferulic acid, p-coumaric; stilbenes; resveratrol; flavonols: (-)-epicatechin, (+)-catechin; flavones: myricetin, quercetin; and 3-*O*-glucosides anthocyanins: Cyanidin, delphinidin, pelargonidin, peonidin, petunidin, malvidin. The most predominant phenolic compounds found in all isolated fractions eluted on XAD and IEX using acetone and methanol were (+)-catechin hydrate and petunidin 3-*O*-glucoside.

Significant differences in phenolic composition of the collected fractions were found using XAD and IEX resins ($P < 0.05$) using acetone and methanol. The fractions eluted on XAD using acetone yielded to fewer collected fractions and higher concentration phenolic compounds compared to methanol using the same resin ($P < 0.05$) (**Table 1**). Fraction VIII had the higher phenolic composition with 476 mg/kg dry matter followed by fraction VII with 394 mg/kg dry matter (**Table 1**). The concentration of total phenolic compounds on XAD using methanol showed the highest collected fractions and there were no significant differences in the phenolic composition found among the collected fractions ($P > 0.05$) (**Table 1**). Fraction XXIII had the higher phenolic composition with 39 mg/kg dry matter followed by fraction XXVI with 37 mg/kg dry matter. However, the rest of the fractions presented close values to each other. On the other hand, there were significant differences in phenolic composition found among the fractions eluted on IEX using acetone and methanol ($P < 0.05$). In addition, the collected fraction using acetone showed a high recovery in fraction IV with 13 mg/kg dry matter compared to the other fractions (**Table 2**). The highest recovery of phenolic compounds on IEX using methanol yielded to 81 mg/kg dry matter in fraction XXX (**Table 2)**. The results showed that fractions eluted on XAD using acetone produced the highest recovery of phenolic compounds in

fractionation. **Figure 1** shows a typical separation of phenolic compounds in fraction VIII isolated on XAD using acetone.

Antibacterial test. The results indicate that phenolic compounds isolated from Cynthiana pomace influence the growth of *E.coli*, *S.aureus* and *L. monocytogenes*. All bacteria strains showed sensitivity, with varying level of inhibition, when tested against grape pomace fractions. The antimicrobial activity of grape pomace (*Vitis aestivalis*) was screened against selected food pathogens (**Table 3** and **4**). Microbial strains had different sensitivities against pomace fractions, and the antimicrobial effect of the studied Cynthiana pomace was variable.

The fraction VIII eluted on XAD using acetone showed higher inhibition against *E.coli* and *L. monocytogenes* compared to other fractions with aAu of 320 respectively (**Table 3**). The aAU for *S.aureus* showed less inhibition at most of the fractions, except for fraction XIX with aAU of 160. In addition, the fractions eluted on XAD using methanol reduced the aAu for *E.coli* and *L. monocytogenes*. In addition, antimicrobial activity for *S.aureus* showed significant differences among the phenolic composition in the fractions eluted on XAD using methanol (P <0.05). Pearson and Spearman test showed that the total concentration of phenolic compounds in all the fractions and aAU were correlated with 0.87 in *S.aureus* strain (**Table 3**).

The fraction III eluted on IEX using acetone showed higher antimicrobial activity for all the strains with an aAU of 160, 320, and 160 respectively (**Table 4**). However, for *E.coli* was the only fraction with activity. Fraction II showed no antimicrobial activity for all the bacteria strains. For *L. monocytogenes* fractions IV, V, and VI showed higher antimicrobial activity compared to *S.aureus* with aAU of 320, 320 and 640 respectively (**Table 4**). In addition, antimicrobial activity for *L. monocytogenes* showed significant differences among the phenolic composition in the fractions eluted on XAD using acetone (P <0.05). Pearson and Spearman test

showed that the total concentration of phenolic compounds in all the fractions and aAU were correlated with 0.98 in *L. monocytogenes* strain (**Table 4**).

The fractions eluted on IEX using methanol showed higher antimicrobial activity for all the strains (**Table 4**). However, fraction XXXIV was the higher with aAU 640 for *E.coli*. Most of the fractions showed antimicrobial activity for *E.coli* and *S.aureus* with aAU of 320, except for fraction XXX with aAU of 160. *L. monocytogenes* showed no antimicrobial activity. There were no significant correlation between the phenolic composition in the different fractions eluted on IEX using acetone and antimicrobial activity.

The bacterial inhibition growth caused by grape pomace extracts can be described by several mechanisms of action. Phenolic compounds can penetrate the semipermeable bacterial membrane where they react with the cytoplasm or cellular proteins. This potential is higher in grape pomace as phenolic acids are present in undissociated form (Paulus 1993). Hydroxycinnamic acids such as ferulic acid, caffeic acid, p-coumaric and anthocyanins such as delphihidin, malvidin, petunidin due to their propenoid side chain, are less polar than the corresponding hydroxybenzoic acids such as gallic acid, and this property facilitates their transport across the cell membrane (Campos 2003) and the mechanism is understood by a neutralization of the membrane electric potential, further penetration of the molecule. Some phenolic compounds can interact with carbohydrates and proteins by hydrogen bonding, hydrophobic and ionic interactions ((McManus 1985). Scalbert (Scalbert 1991) proposed that the antibacterial activity of tannins could be due to the inhibition of extracellular microbial enzymes. Moreover, the complexing of metal ions from the bacterial growth environment could also be a possible mechanism for their antimicrobial properties. The lipidic wall of Gram negative bacteria is characterized as great barrier for extracted phenolic compounds to get into

the cytoplasm and our findings with *E.coli* are in agreement with the findings of Puupponen-Pimiä (Puupponen-Pimiä 2001; Puupponen-Pimiä 2005a; Puupponen-Pimiä 2005b).

Conclusion

In conclusion, phenolic extracts of grape pomace (*Vitis aestivalis)* inhibited the growth of select foodborne pathogens. The XAD and IEX resin provide an effective method for removing sugars from natural products and provide a first step in purifying and isolating phenolic compounds. Further investigations are required to elucidate the exact nature of the inhibitory factor(s), in terms of optimum phenolic weight and composition in the fractions isolated from the resins.

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Table 1. Content of phenolic compounds in pigmented fractions using XAD resin (mg/kg dry matter)

Amberlite XAD	
Acetone	**TOTALS ± SEM**
Fraction VII	394.55 ± 36.38[a]
Fraction VIII	476.04 ± 13.74[a]
Fraction XIX	302.45 ± 10.54[b]
Fraction X	40.15 ± 1.55[c]
Fraction XI	75.55 ± 2.34[c]
Methanol	**TOTALS ± SEM**
Fraction XXIII	39.15 ± 1.16[a]
Fraction XXIV	29.87 ± 0.88[a]
Fraction XXV	29.31 ± 0.87[a]
Fraction XXVI	37.43 ± 0.99[a]
Fraction XXVII	26.45 ± 0.60[a]
Fraction XXVIII	28.61 ± 0.55[a]

[a-c] Means with similar letter are not significantly different (Tukey, $P > 0.05$)

Table 2. Content of phenolic compounds in pigmented fractions using IEX resin (mg/kg dry matter

Ion Exchange	
Acetone	**TOTALS ± SEM**
Fraction II	5.58 ± 1.77[a]
Fraction III	6.91 ± 1.55[a]
Fraction IV	13.31 ± 1.38[a]
Fraction V	2.55 ± 0.41[a]
Fraction VI	3.40 ± 0.63[a]
Methanol	**TOTALS ± SEM**
Fraction XXX	81.30 ± 3.13[a]
Fraction XXXI	57.73 ± 2.12[b]
Fraction XXXII	64.39 ± 2.40[b]
Fraction XXXIII	41.13 ± 1.96[c]
Fraction XXXIV	41.83 ± 2.03[c]

[a,b] Means with similar letter are not significantly different (Tukey, $P > 0.05$)

Table 3. Activity units (aAU/mL) of the pigmented fraction isolated from grape pomace (*Vitis aestivalis*)

Amberlite XAD					
Acetone					
Bacterial species	**Fraction VII**	**Fraction VIII**	**Fraction IX**	**Fraction X**	**Fraction XI**
E.coli O157:H7	160	320	320	ND	ND
Staphylococcus aureus	80	80	160	320	320
Listeria monocytogenes	160	320	160	160	160

Methanol						
Bacterial species	**Fraction XXIII**	**Fraction XXIV**	**Fraction XXV**	**Fraction XXVI**	**Fraction XXVII**	**Fraction XXVIII**
E.coli O157:H7	80	80	160	80	80	80
Staphylococcus aureus	160	160	80	160	80	80
Listeria monocytogenes	160	160	80	160	160	160

Table 4. Activity units (aAU/mL) of the pigmented fraction isolated from grape pomace (*Vitis aestivalis*)

Ion Exchange					
Acetone					
Bacterial species	**Fraction II**	**Fraction III**	**Fraction IV**	**Fraction V**	**Fraction VI**
E.coli O157:H7	ND	160	ND	ND	ND
Staphylococcus aureus	ND	320	160	160	160
Listeria monocytogenes	ND	160	320	80	640
Methanol					
Bacterial species	**Fraction XXX**	**Fraction XXXI**	**Fraction XXXIV**	**Fraction XXXV**	**Fraction XXXVI**
E.coli O157:H7	160	320	320	320	640
Staphylococcus aureus	160	320	320	320	320
Listeria monocytogenes	ND	ND	ND	ND	ND

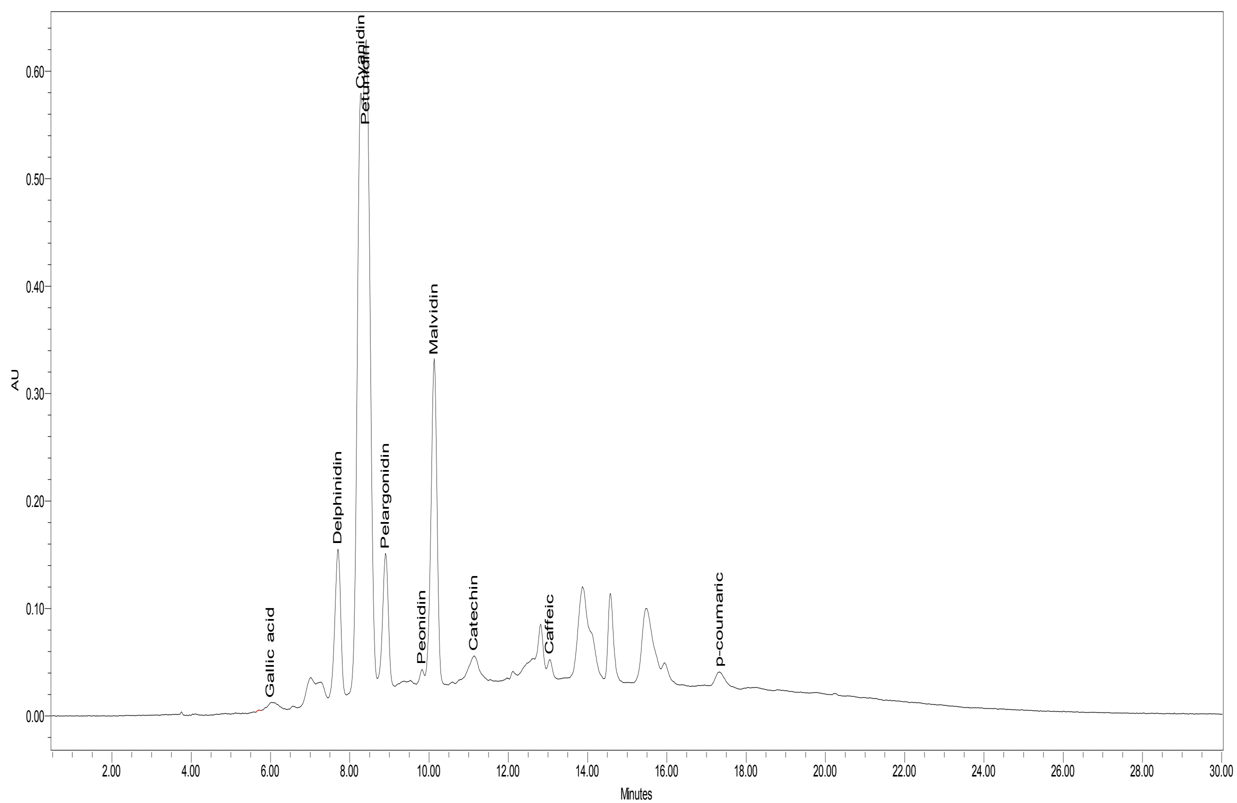


Figure 1. Typical separation of phenolic compounds eluted on XAD using acetone (fraction VIII)

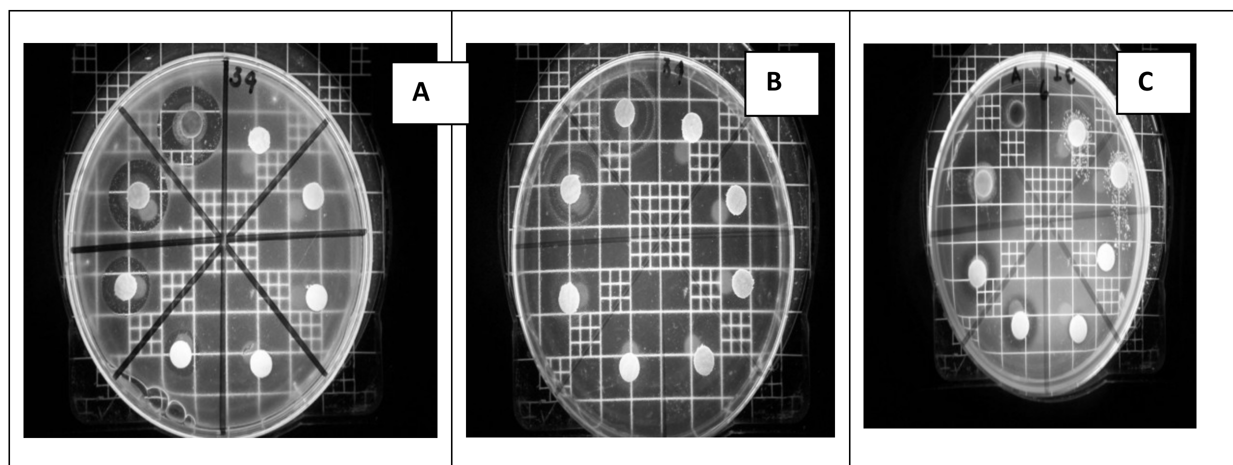


Figure 2. Inhibitory activity of phenolic compounds against bacteria strains

(A) Antimicrobial activity towards *E. coli* of fraction XXXIV eluted on IEX using methanol

(B) Antimicrobial activity towards *S.aureus* of fraction XXXIV eluted on IEX using methanol

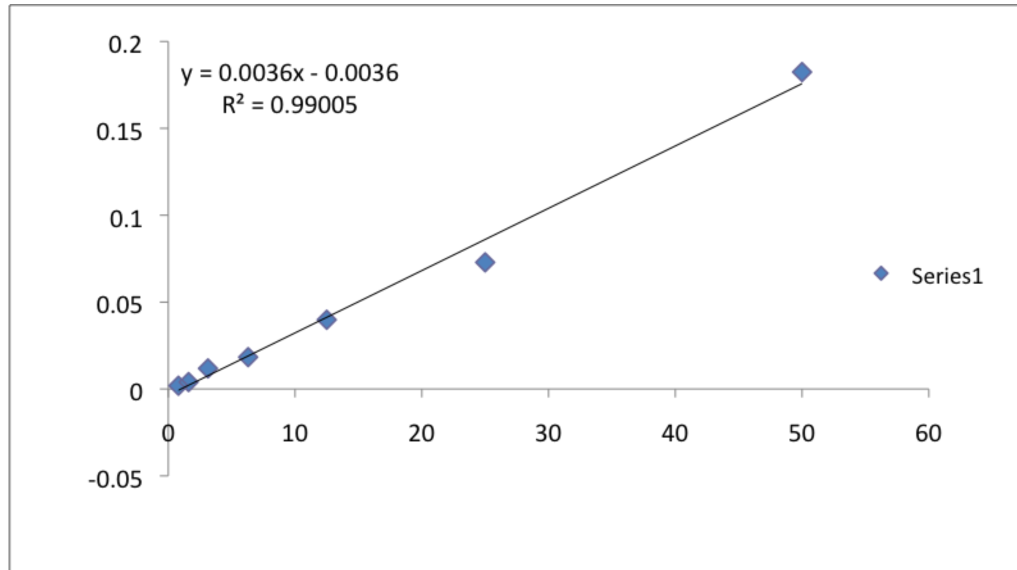
(C) Antimicrobial activity towards *L. monocytogenes* of fraction V I eluted on IEX using acetone

APPENDICES

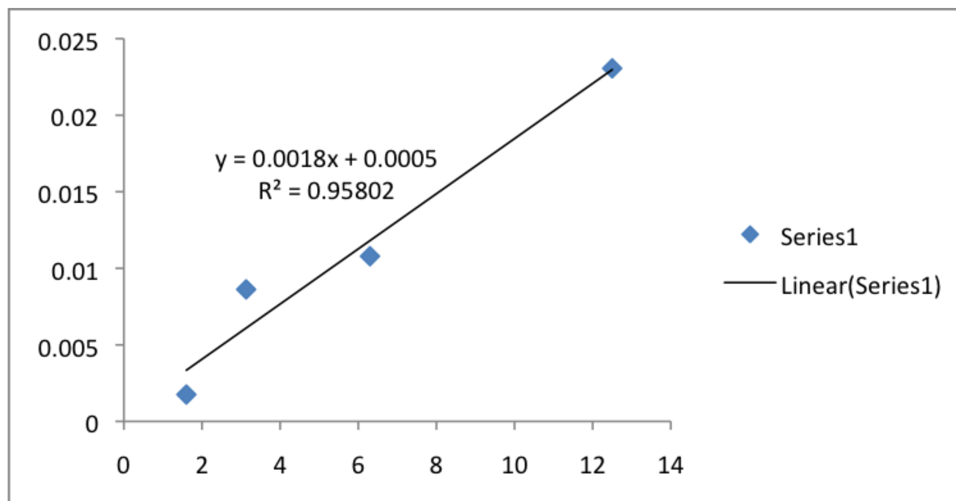
APPENDICE A

INTERNAL CALIBRATION CURVES OF THE STANDARDS BY HPLC

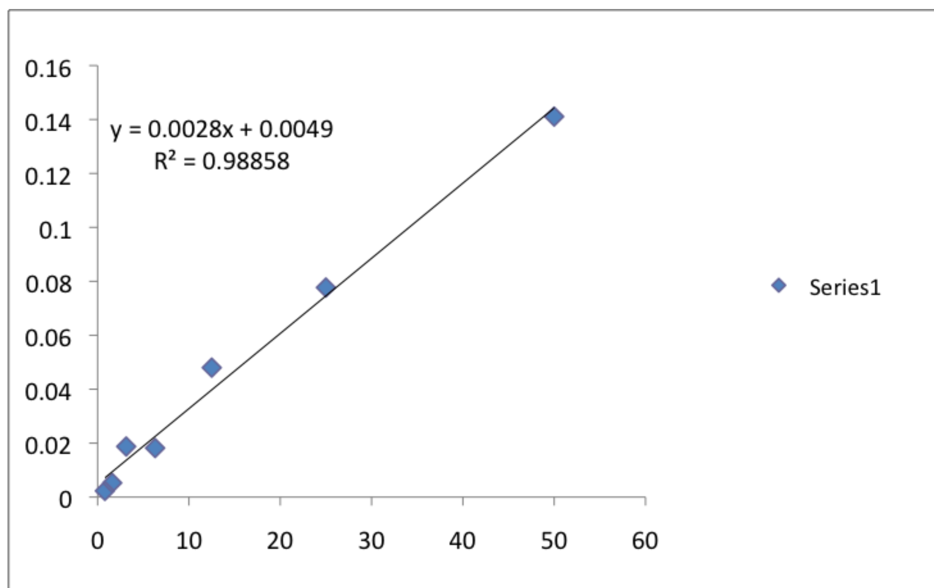
Gallic acid



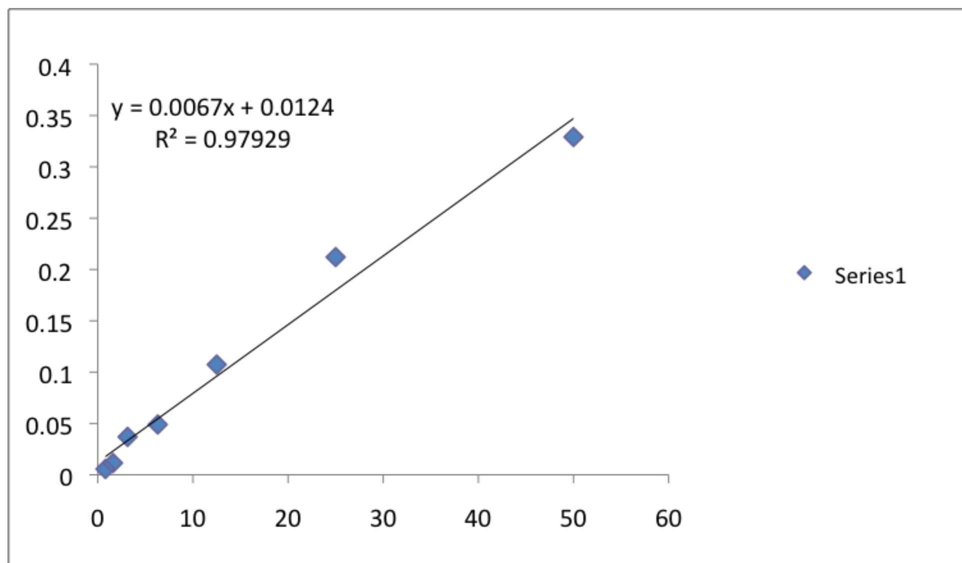
(+) Catechin hydrate



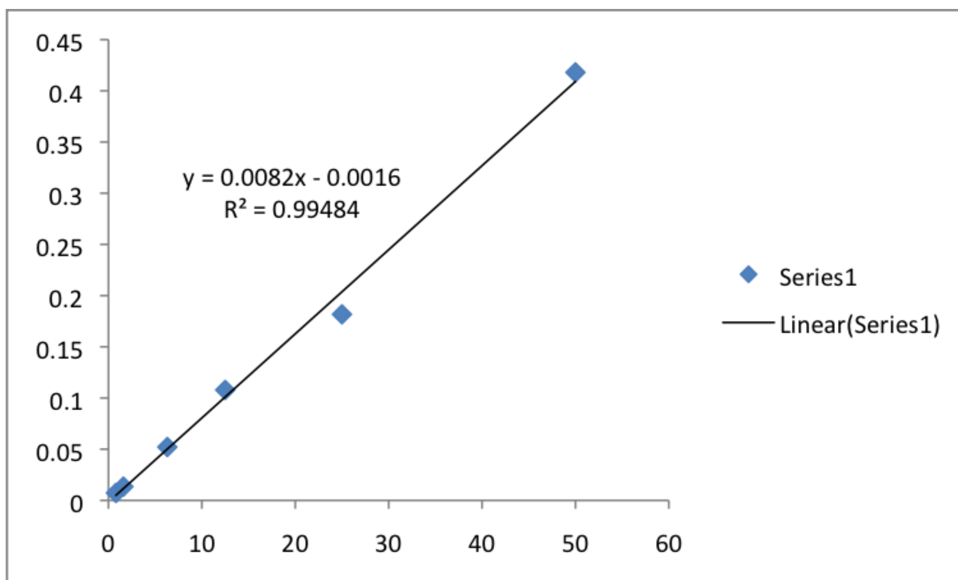
(-)Epicathecin gallate



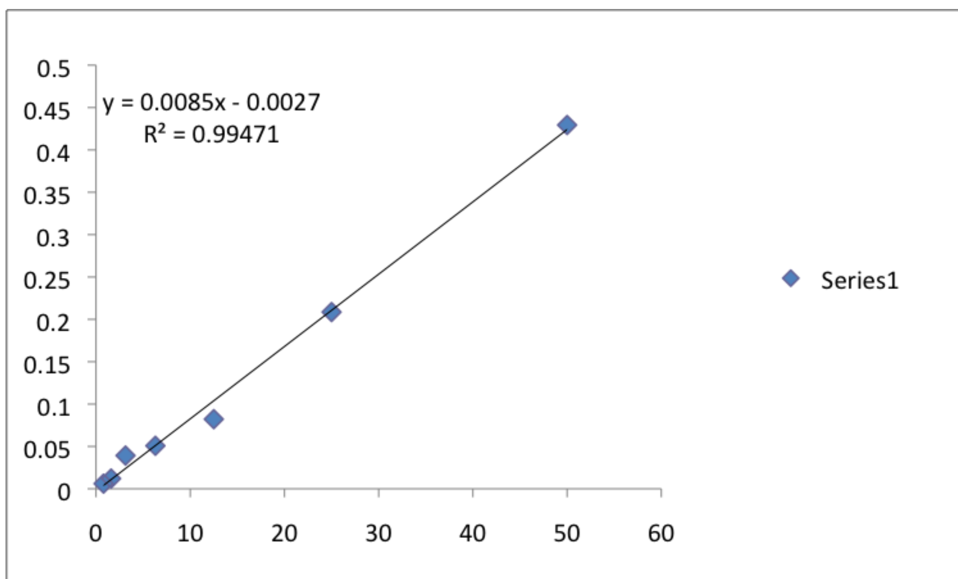
Caffeic acid



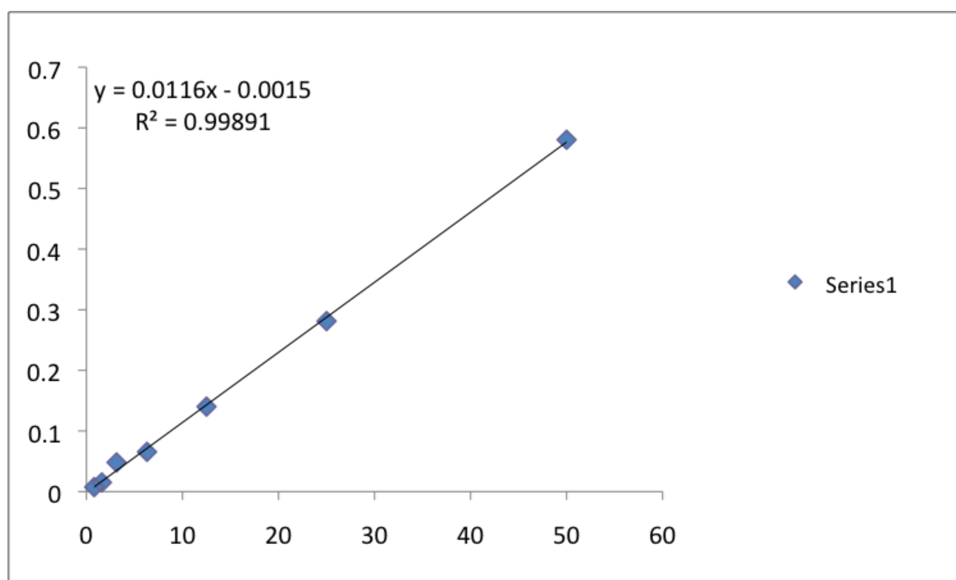
p-coumaric acid



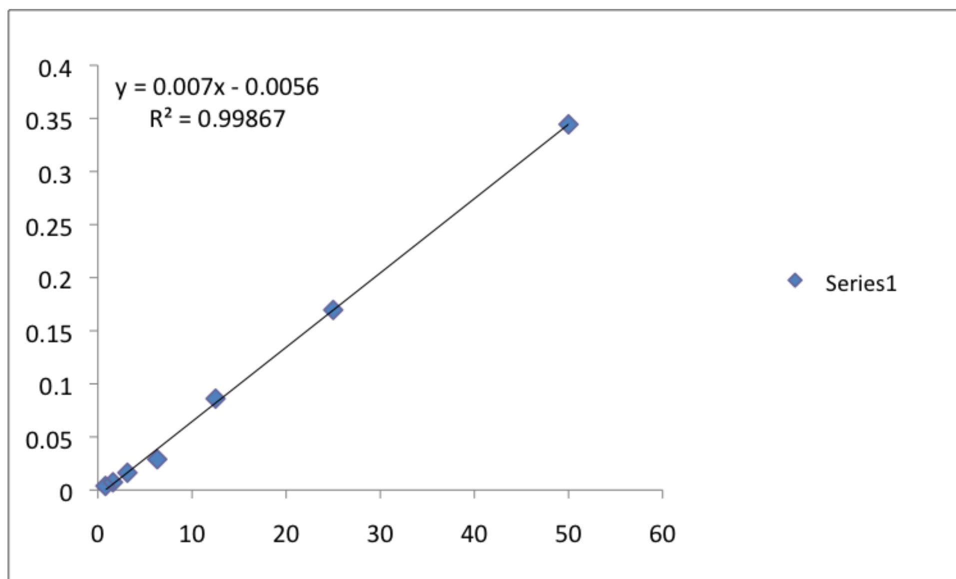
Ferulic acid



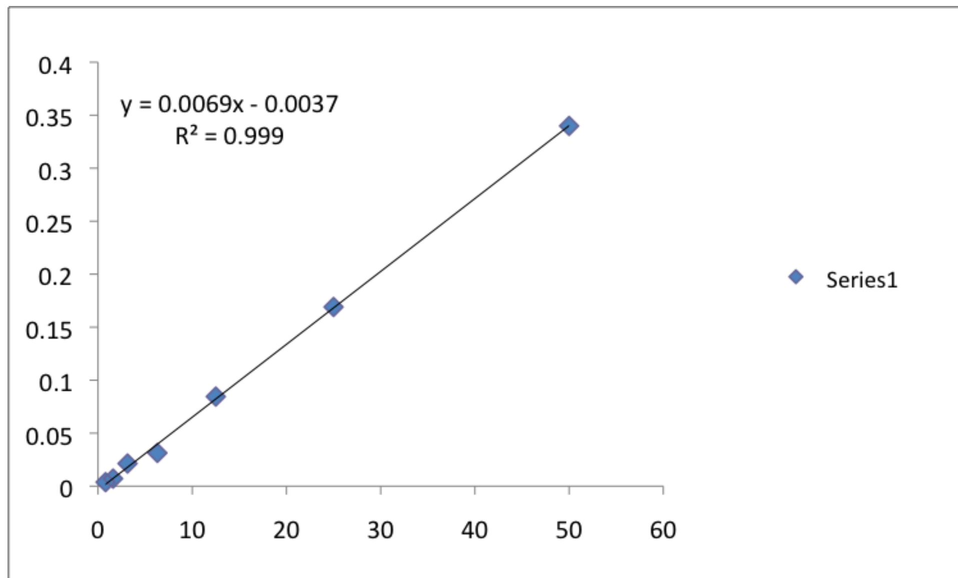
Resveratrol



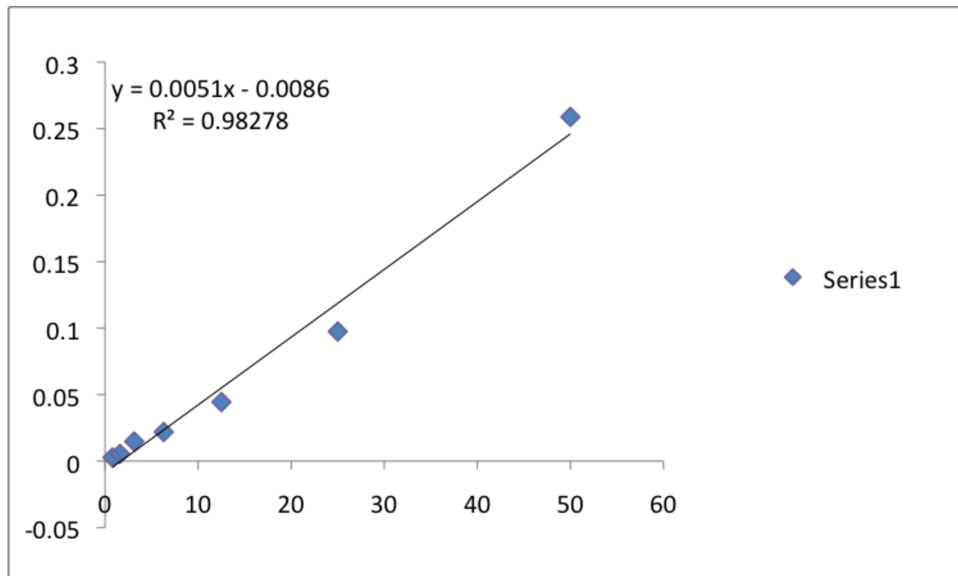
Myricetin



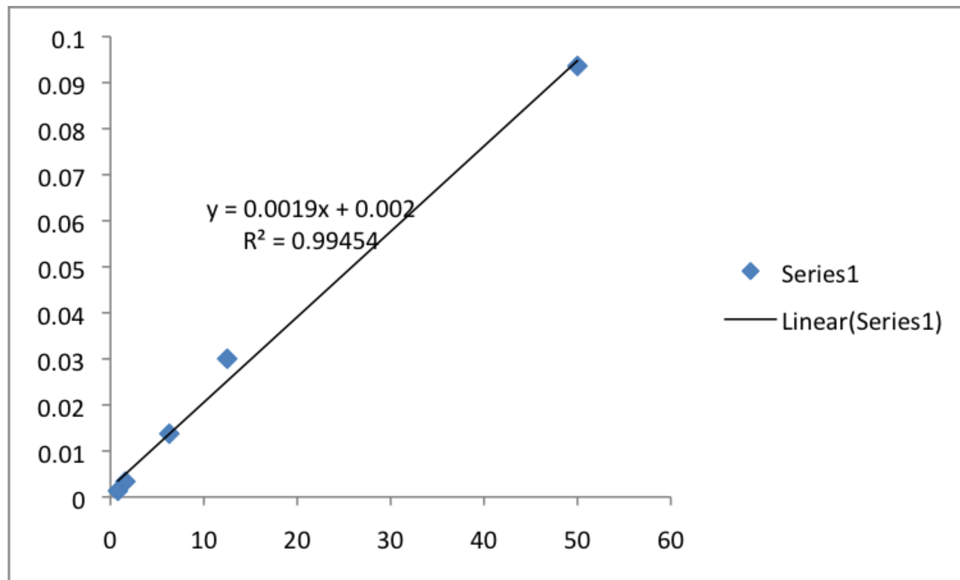
Quercetin



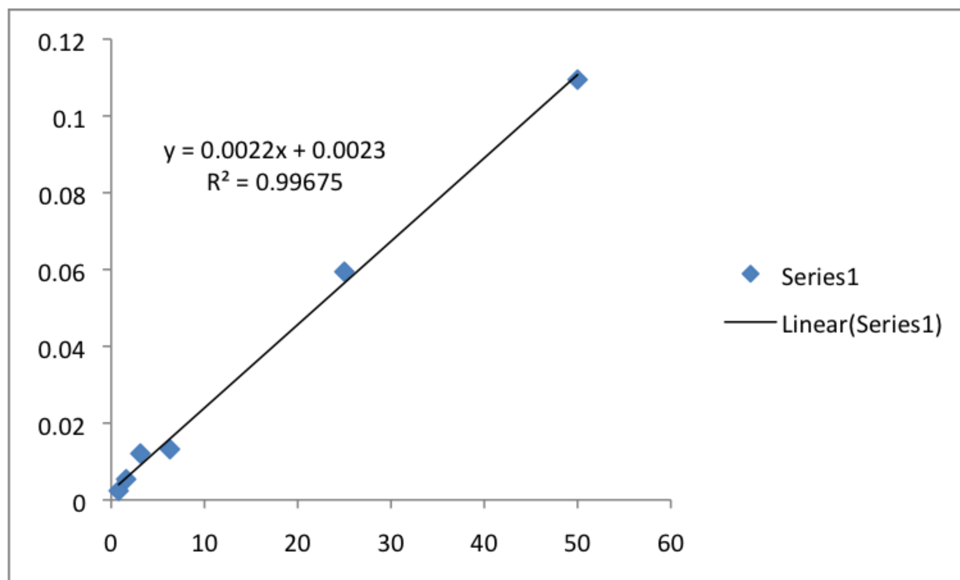
Kaempferol



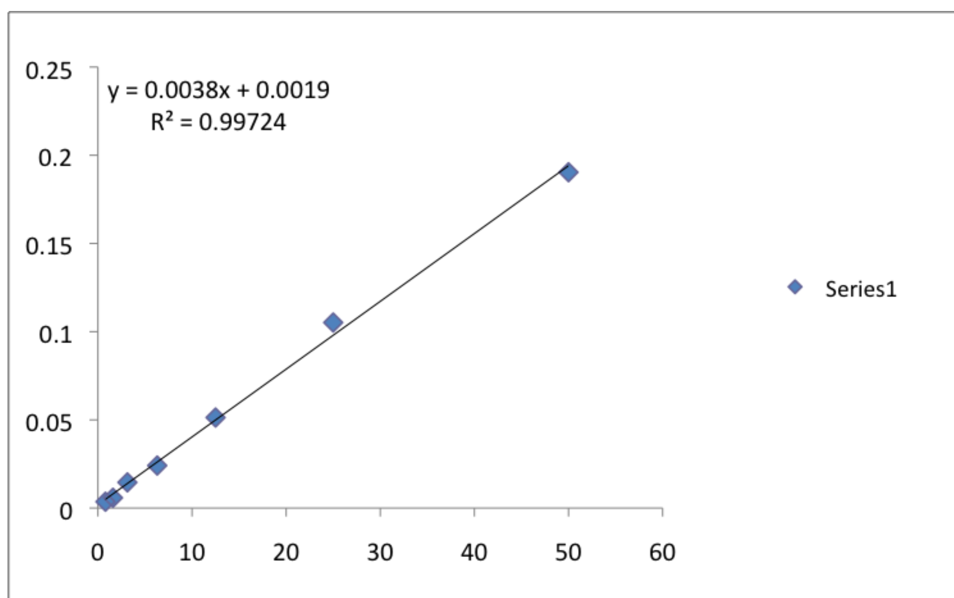
Isoharmnetin



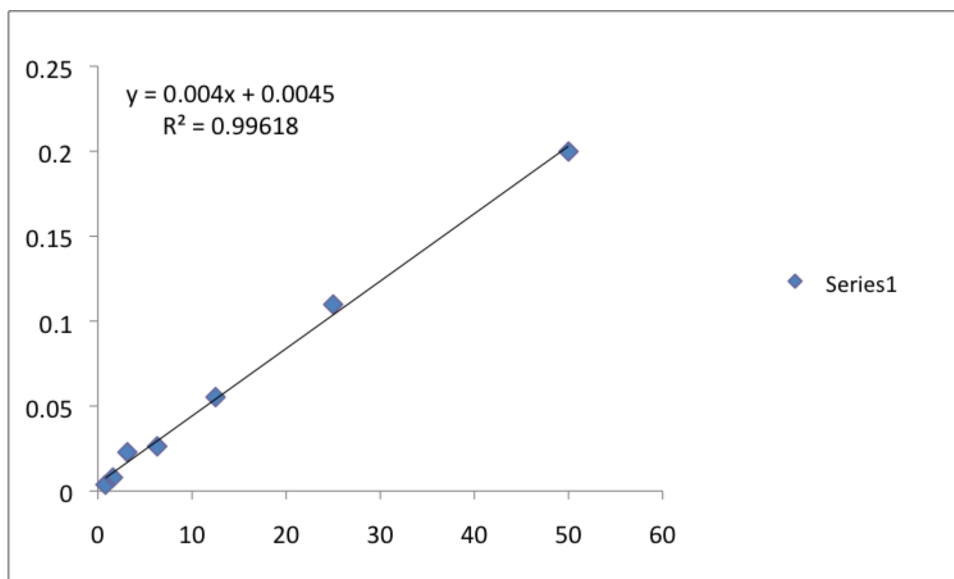
Delphinidin



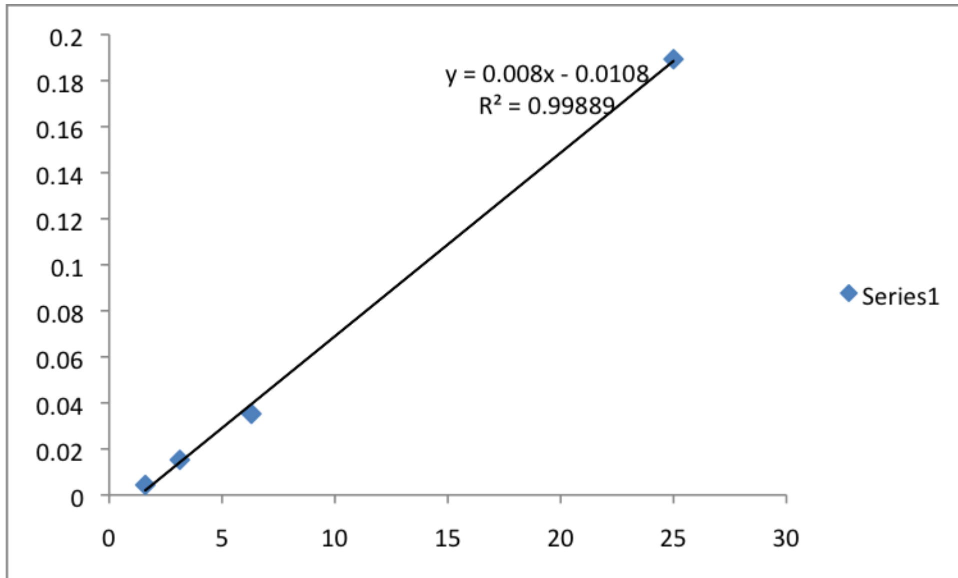
Cyanidin



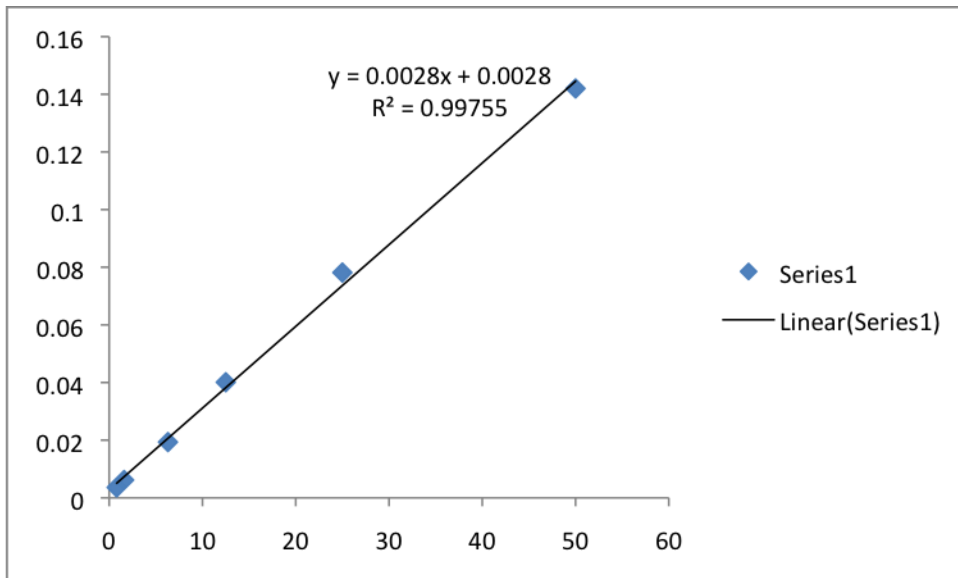
Petunidin



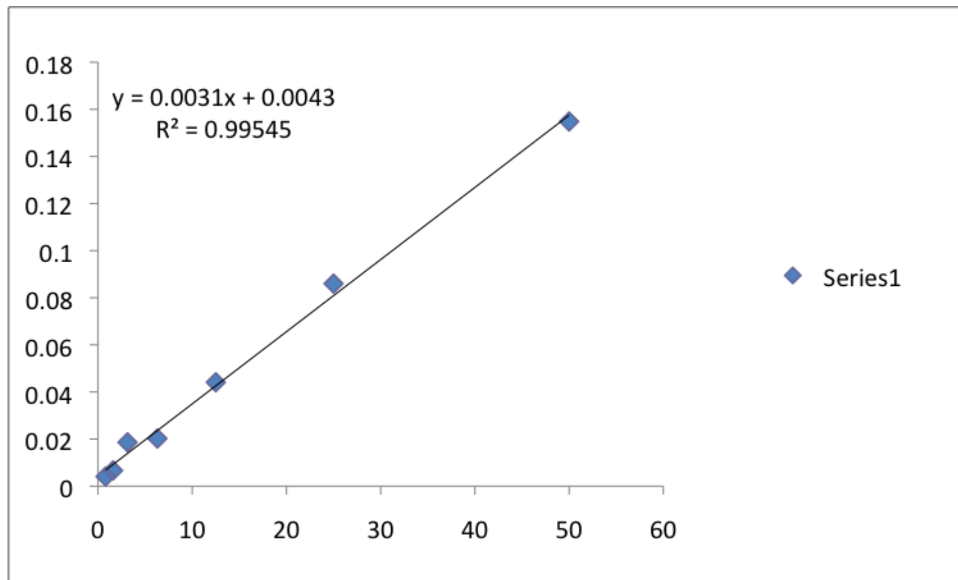
Pelargonidin



Peonidin



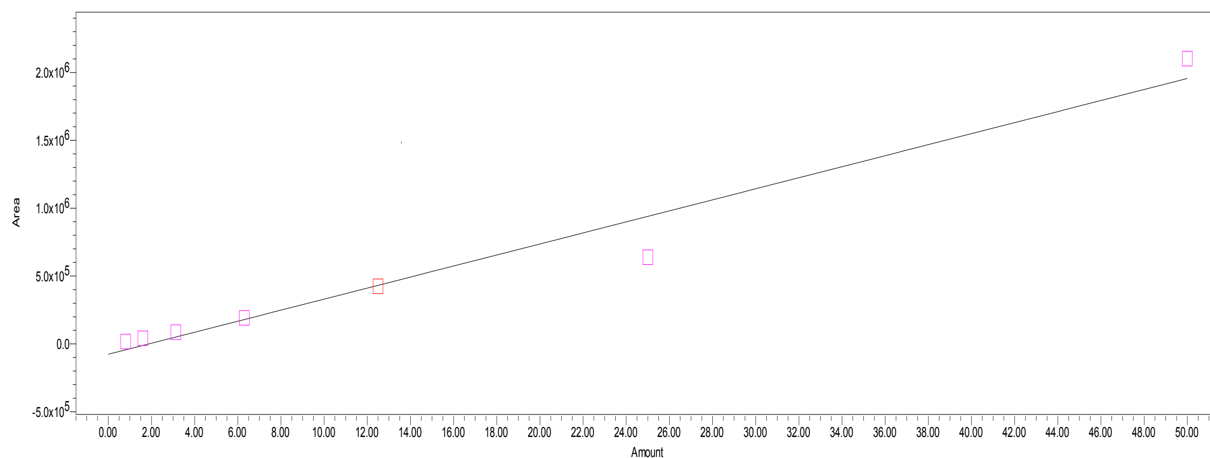
Malvidin



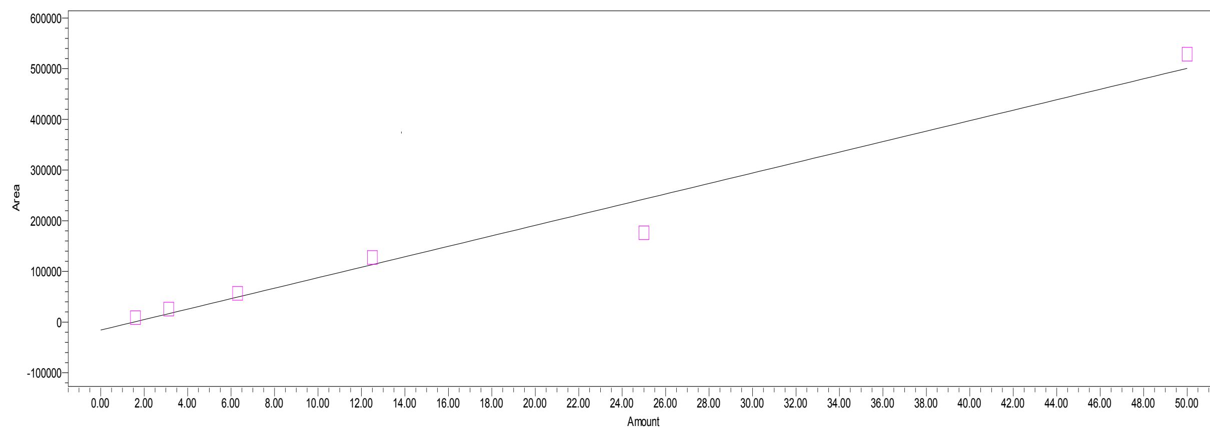
APPENDICE B

EXTERNAL CALIBRATION CURVES OF THE STANDARDS BY HPLC

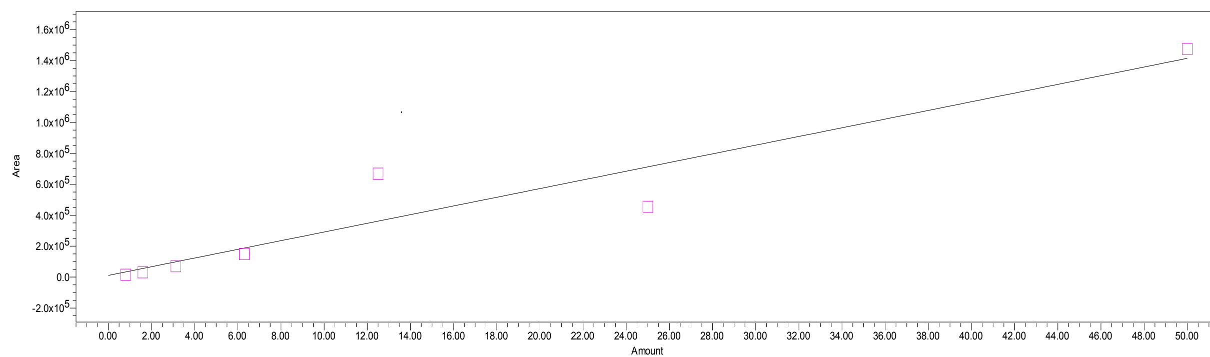
Gallic acid



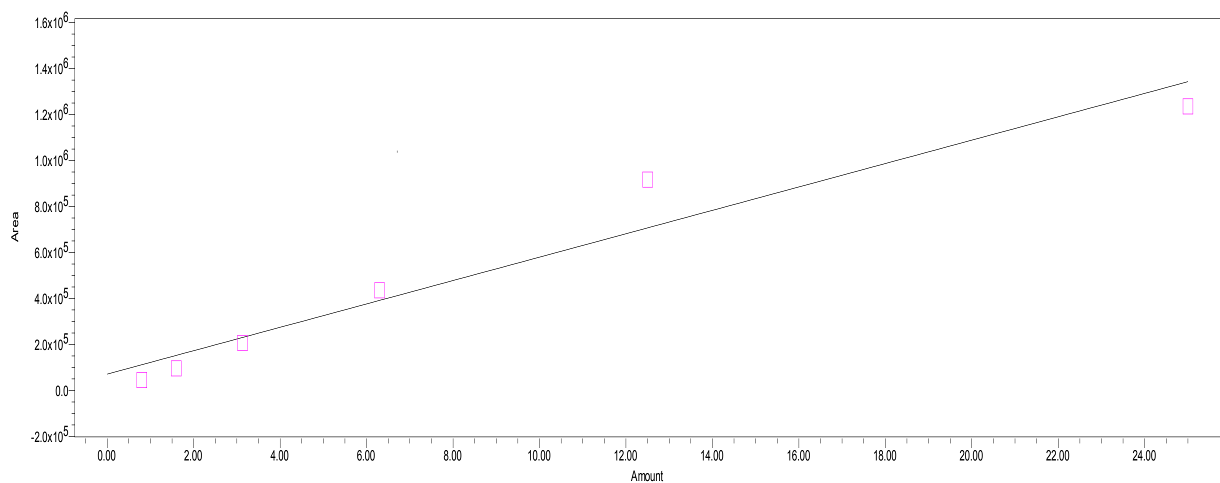
(+) Catechin hydrate



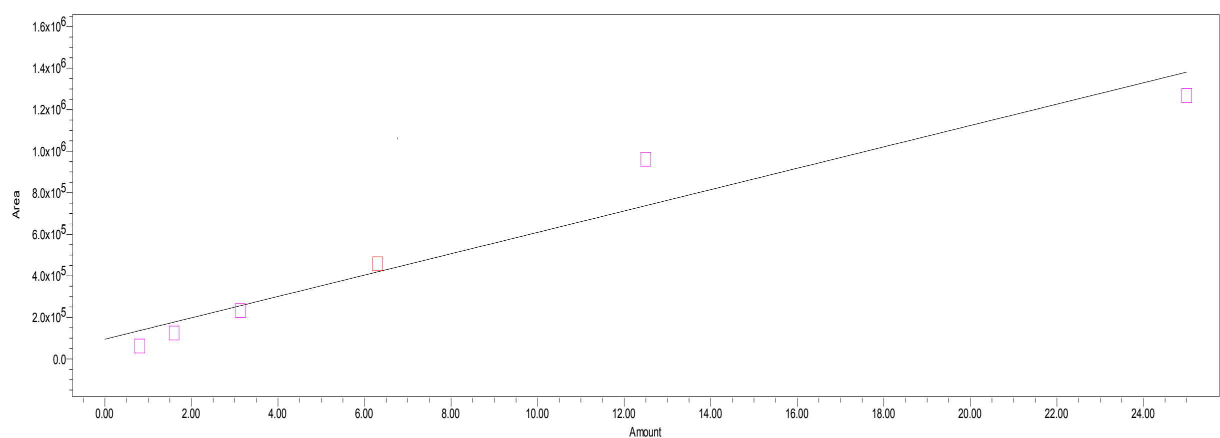
(-) Epicatechin gallate



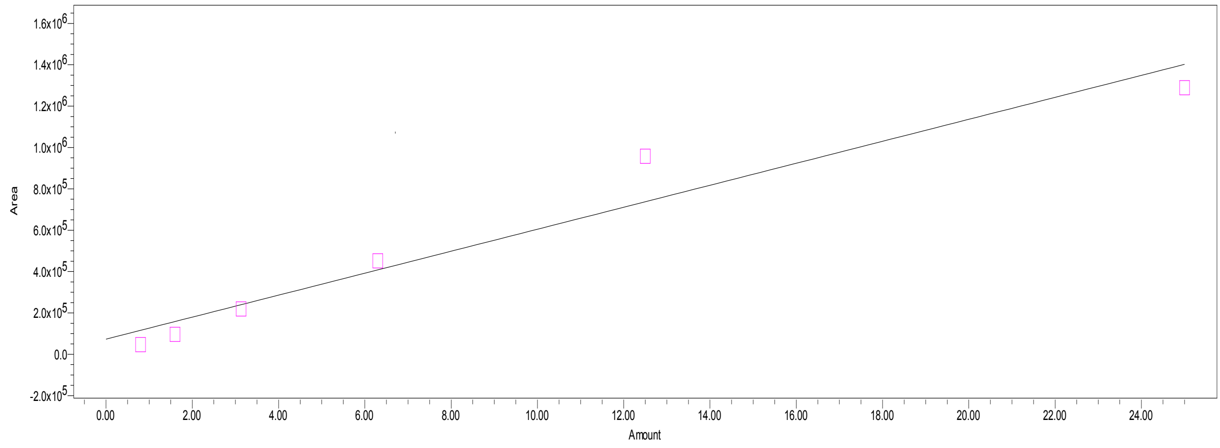
Caffeic acid



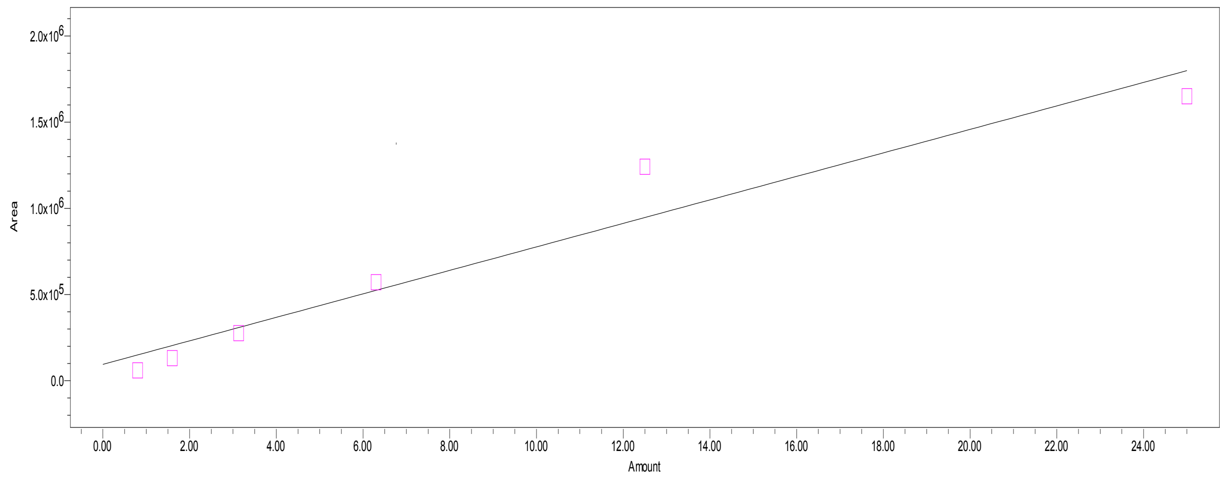
p-coumaric acid



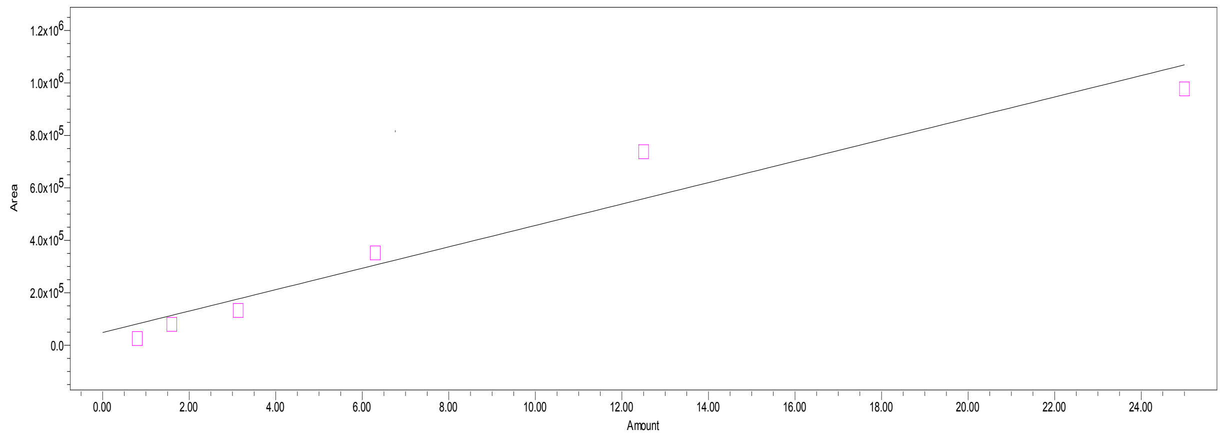
Ferulic acid



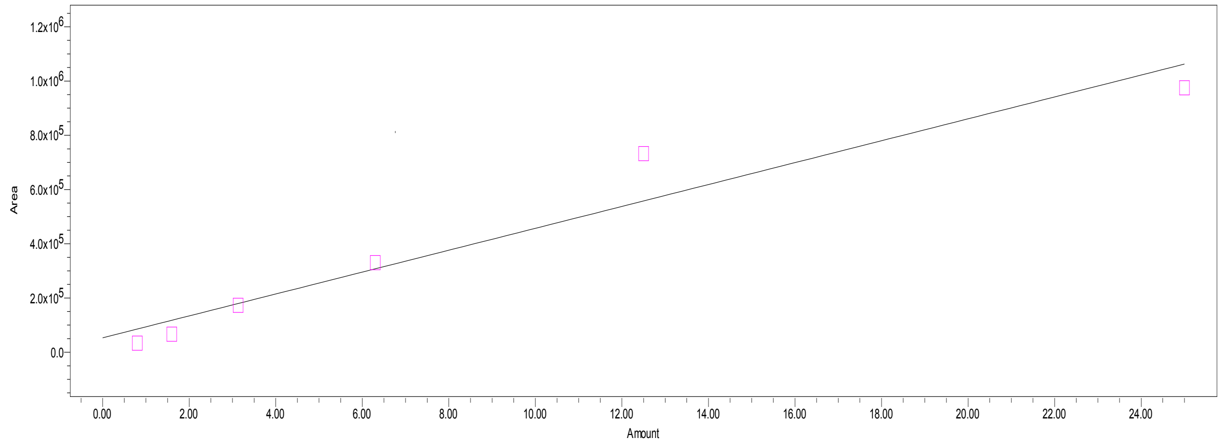
Resveratrol



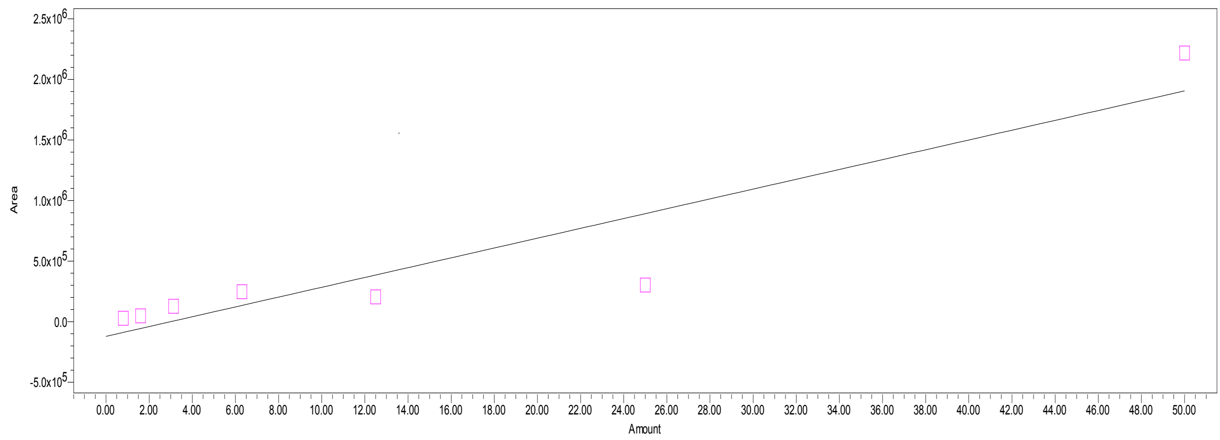
Myricetin



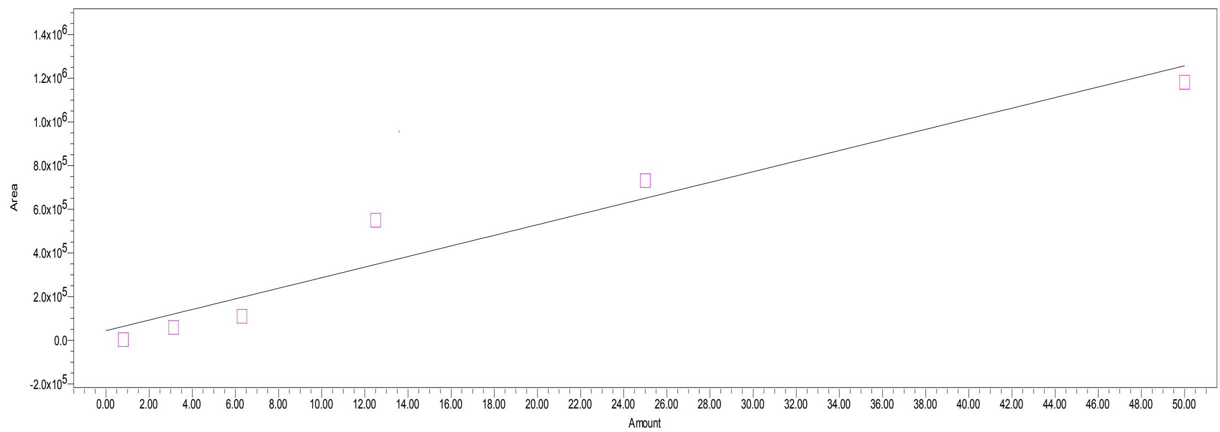
Quercetin



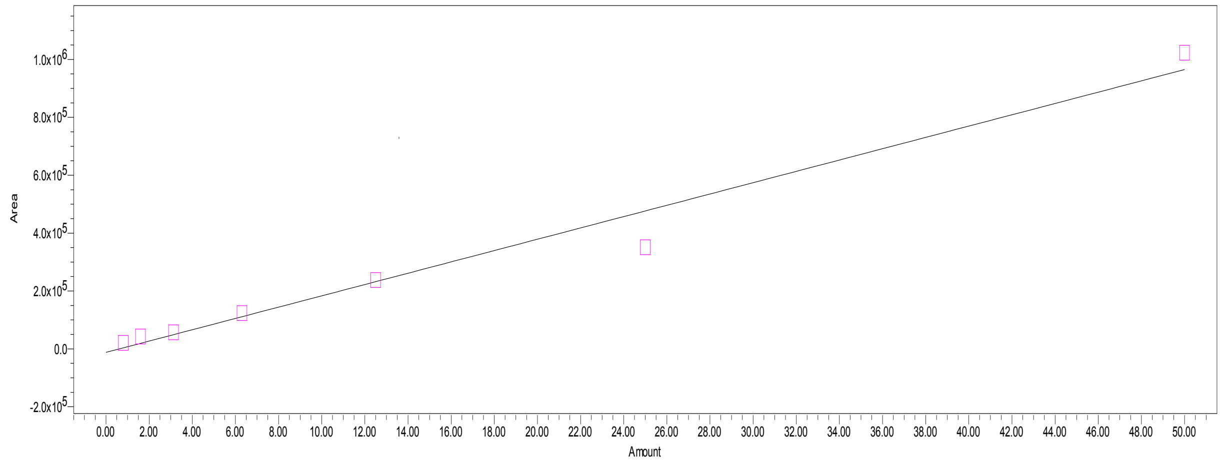
Kaempferol



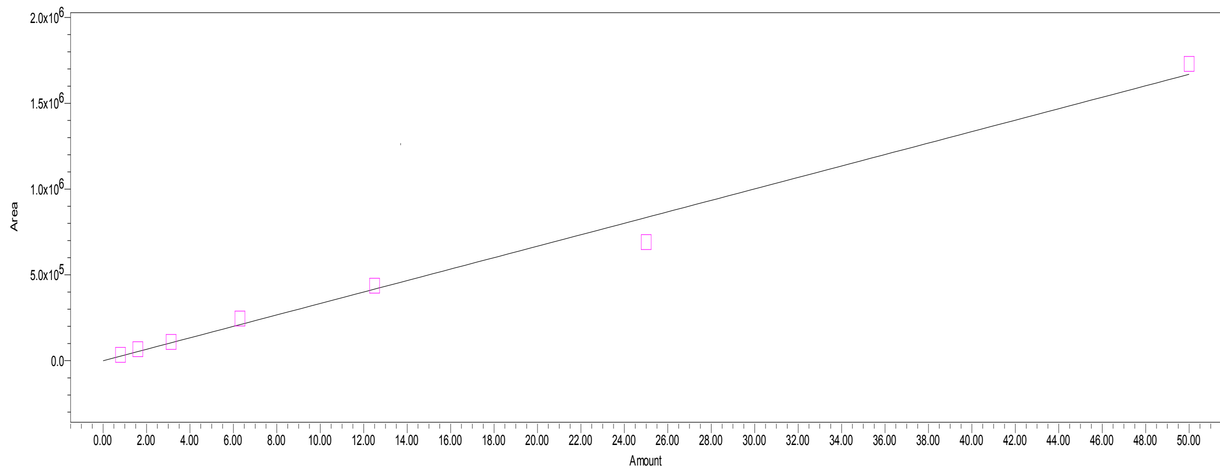
Isoharmnetin



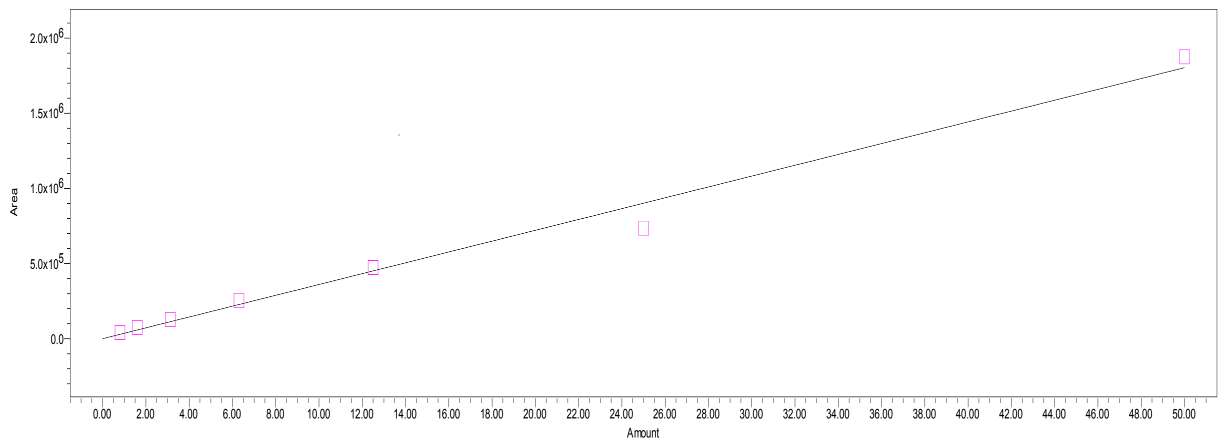
Delphinidin 3-*O*-glucoside



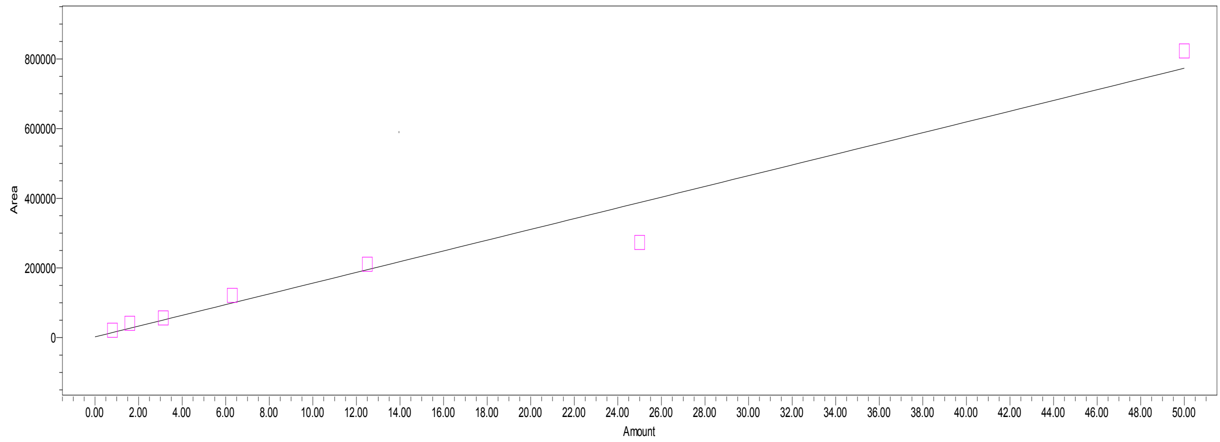
Cyanidin 3-*O*-glucoside



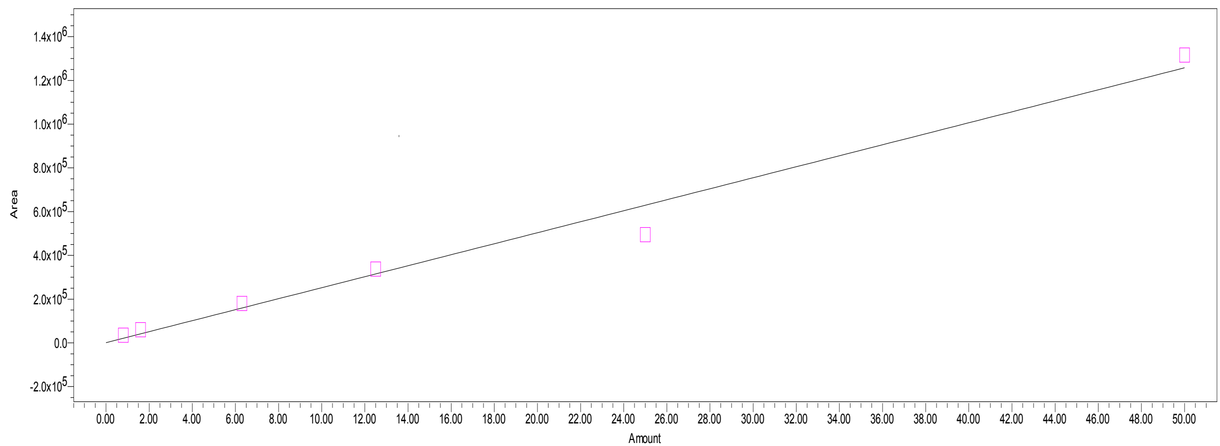
Petunidin 3-*O*-glucoside



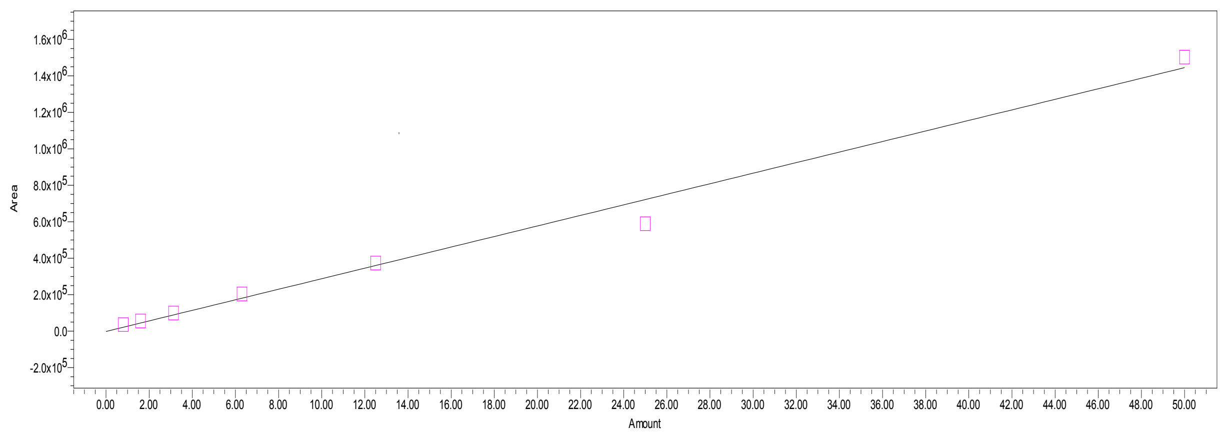
Pelargonidin 3-*O*-glucoside



Peonidin 3-*O*-glucoside



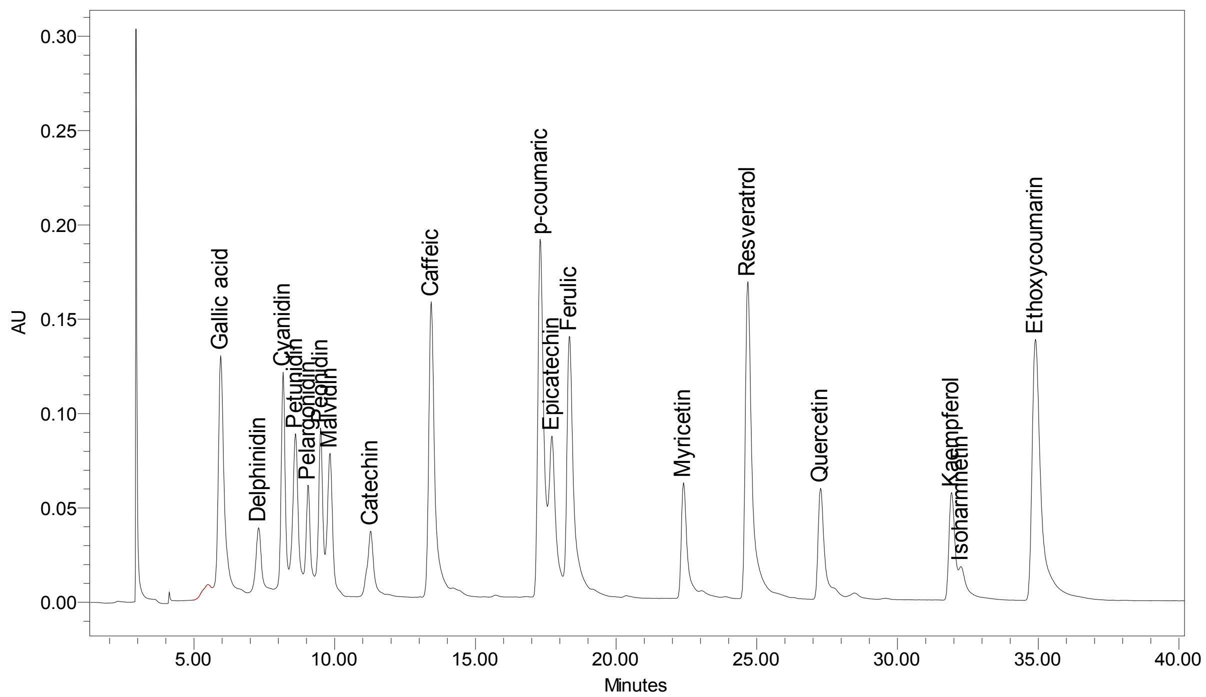
Malvidin 3-*O*-glucoside



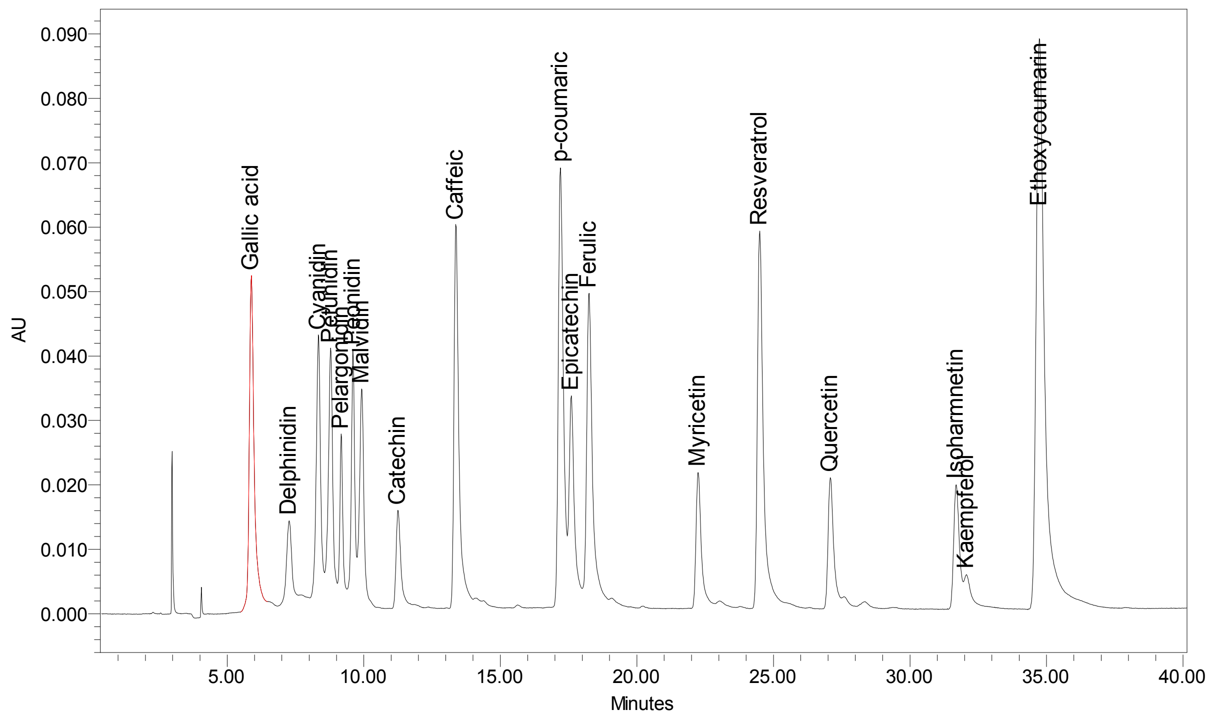
APPENDICE C

TYPICAL SEPARATION OF STANDARDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

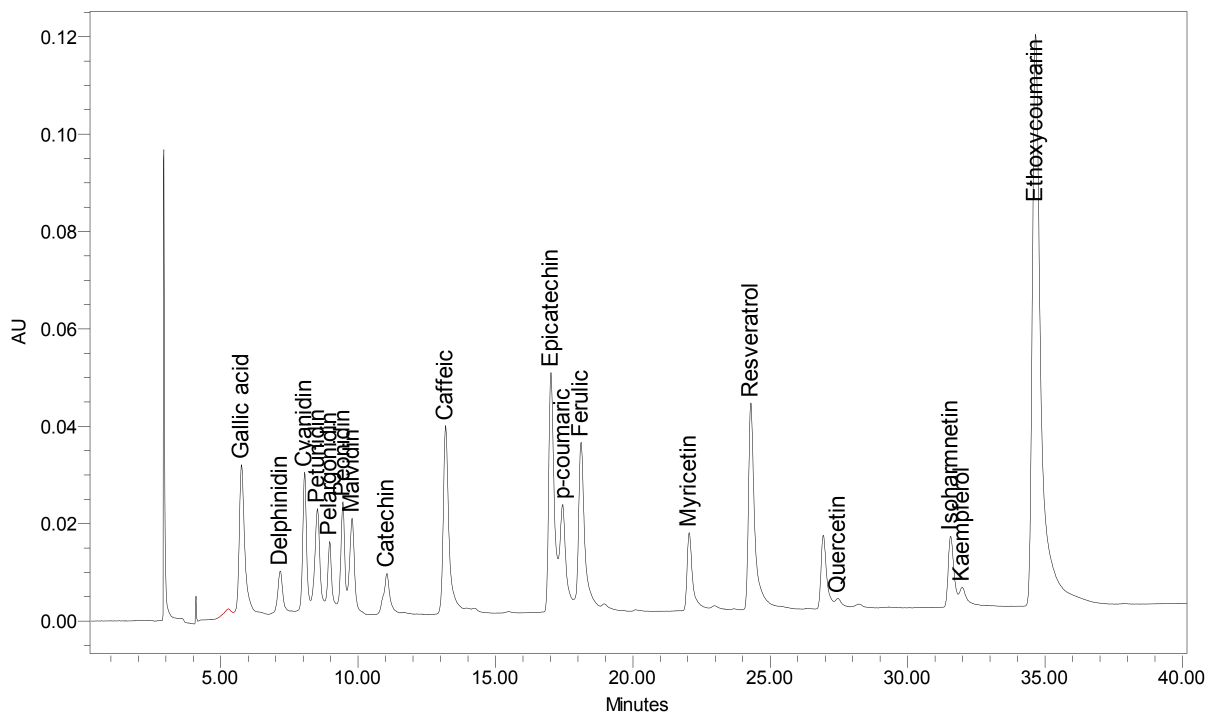
50 ppm



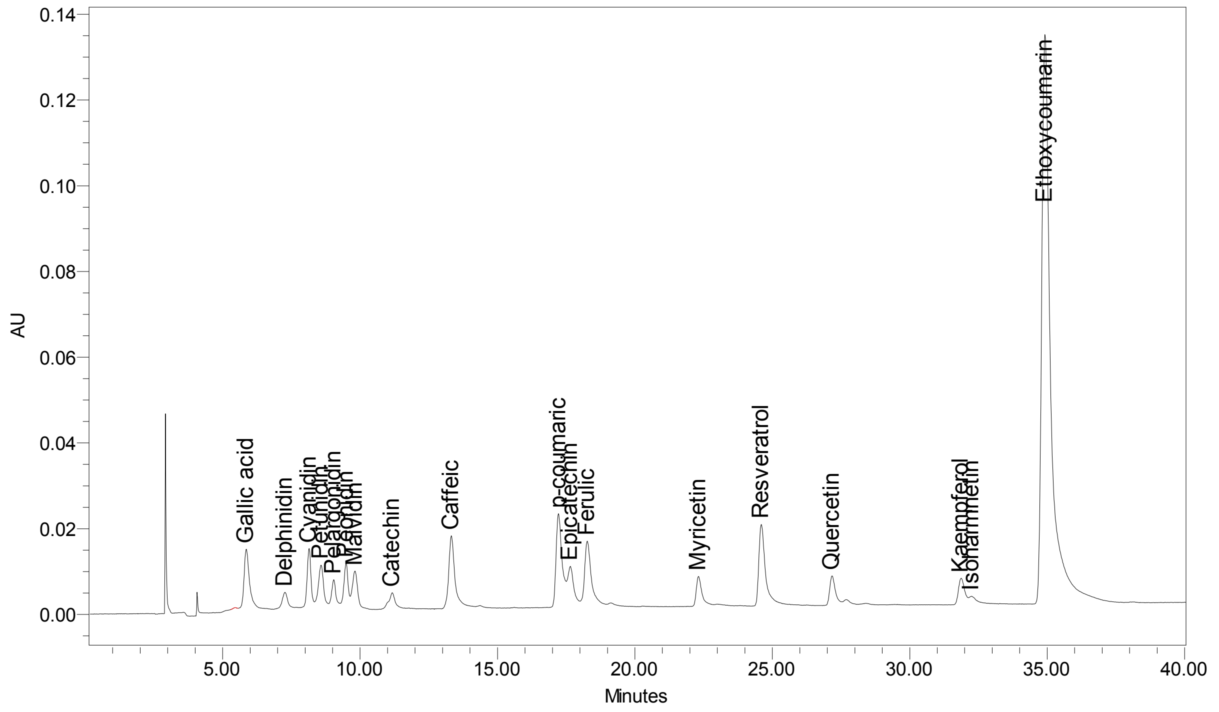
25 ppm



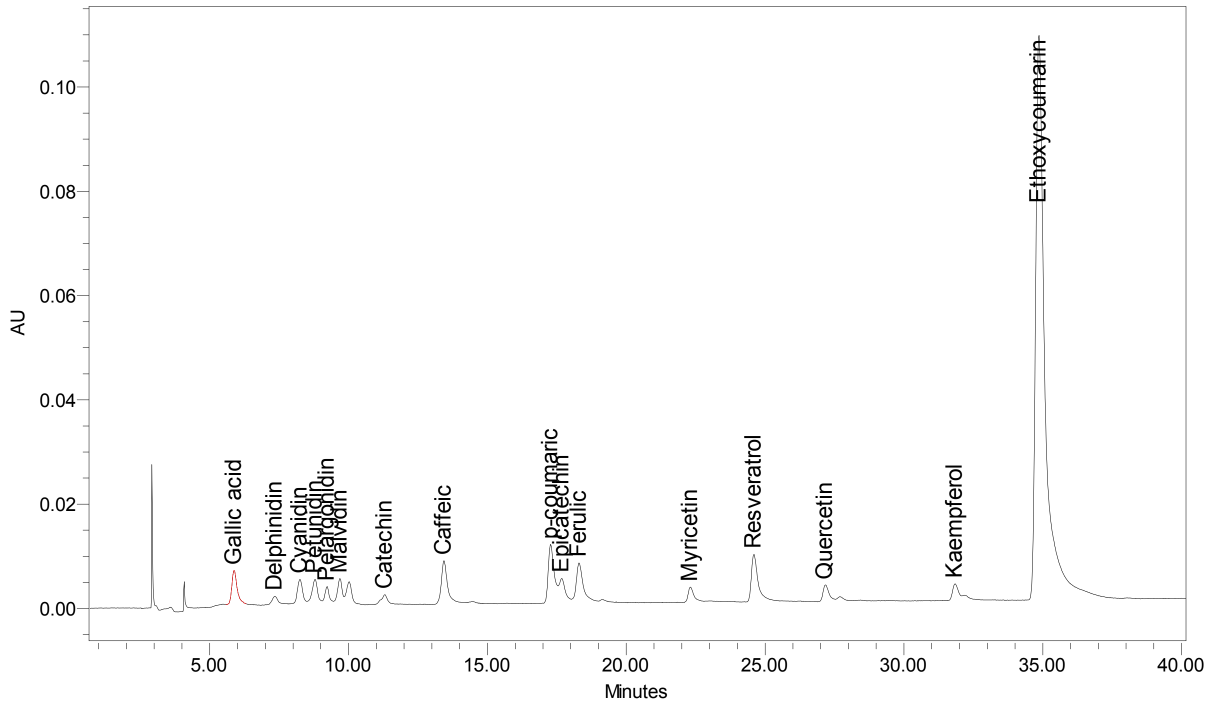
12.50 ppm



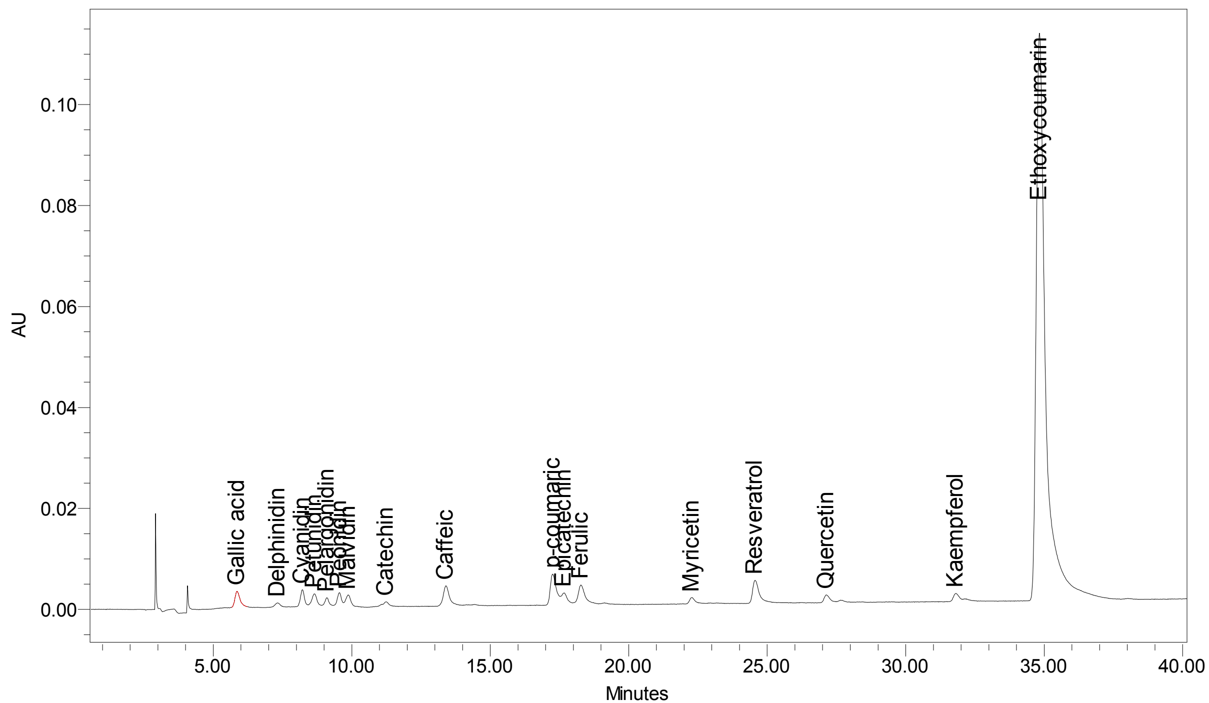
6.30 ppm



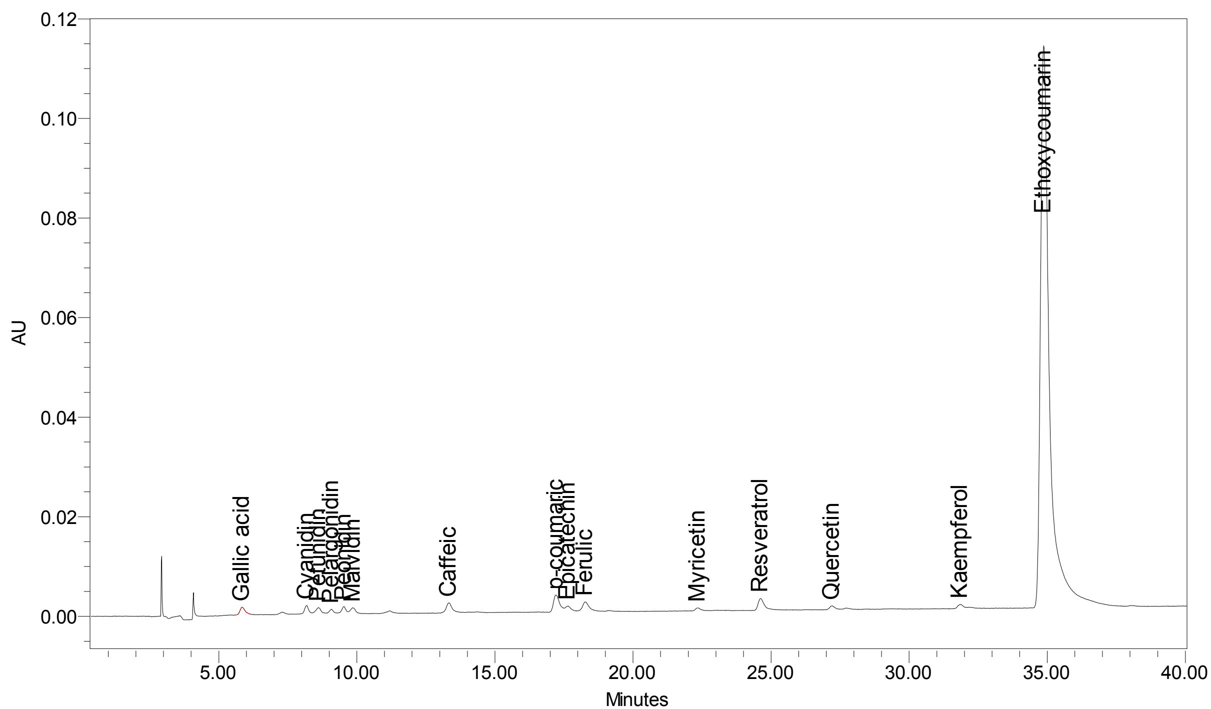
3.13 ppm



1.60 ppm



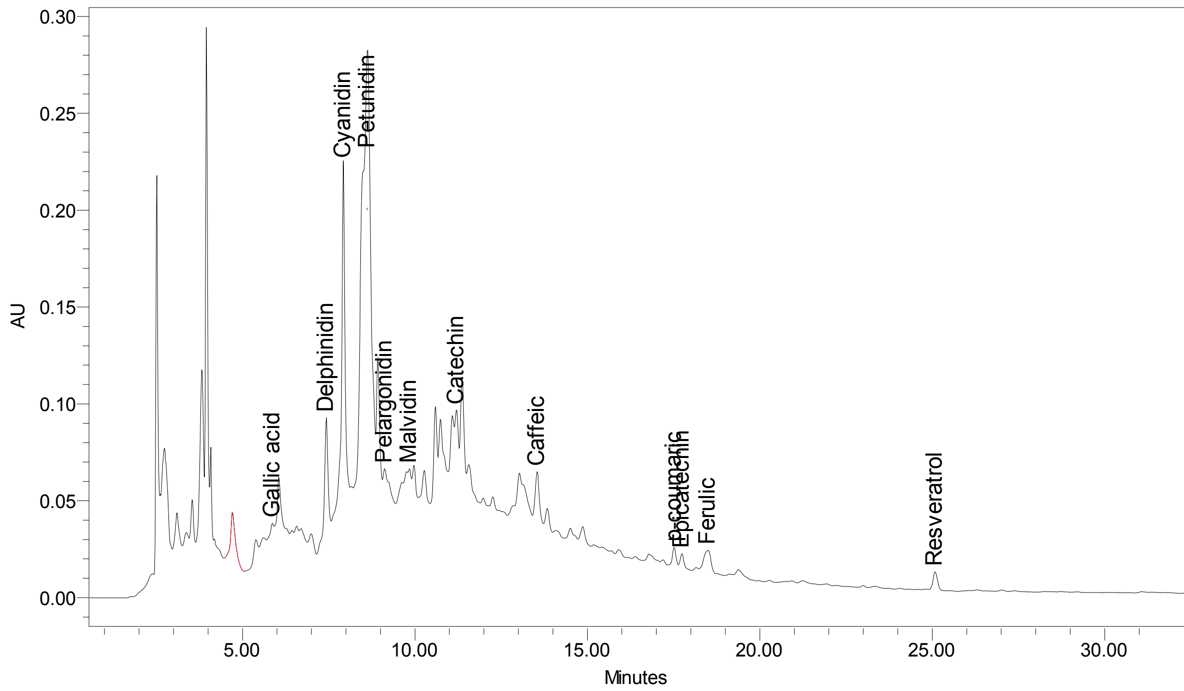
0.80 ppm



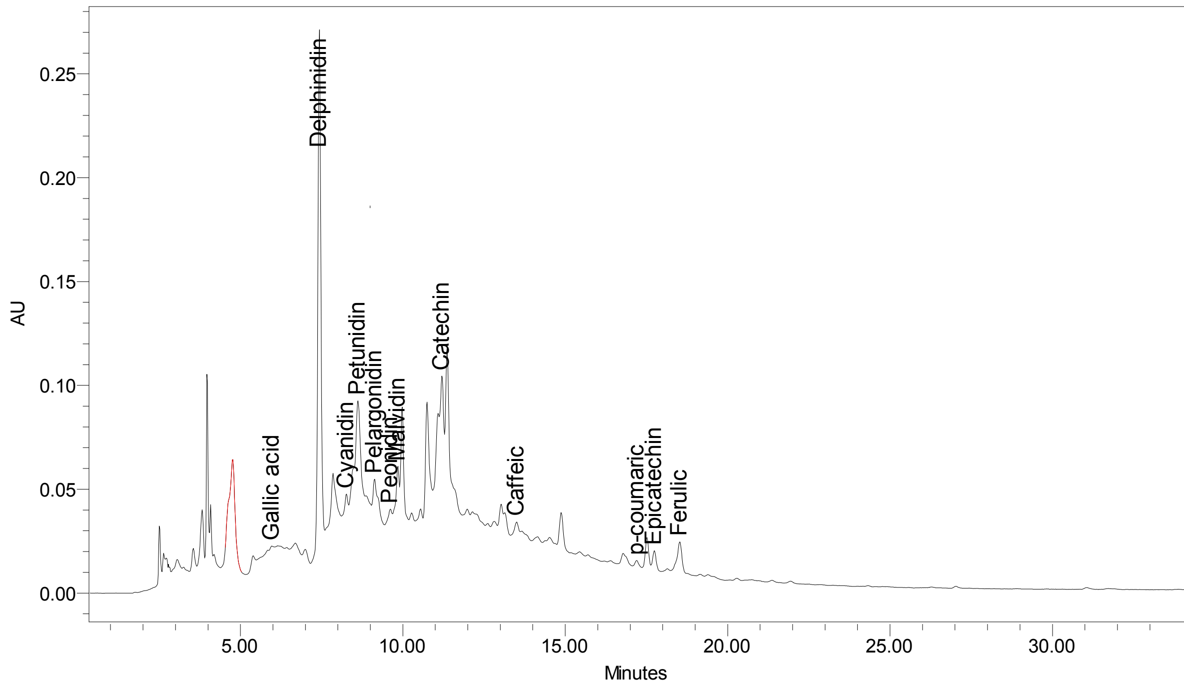
APPENDICE D

CHROMATOGRAMS OF JUICE, WHOLE GRAPE AND POMACE SAMPLES

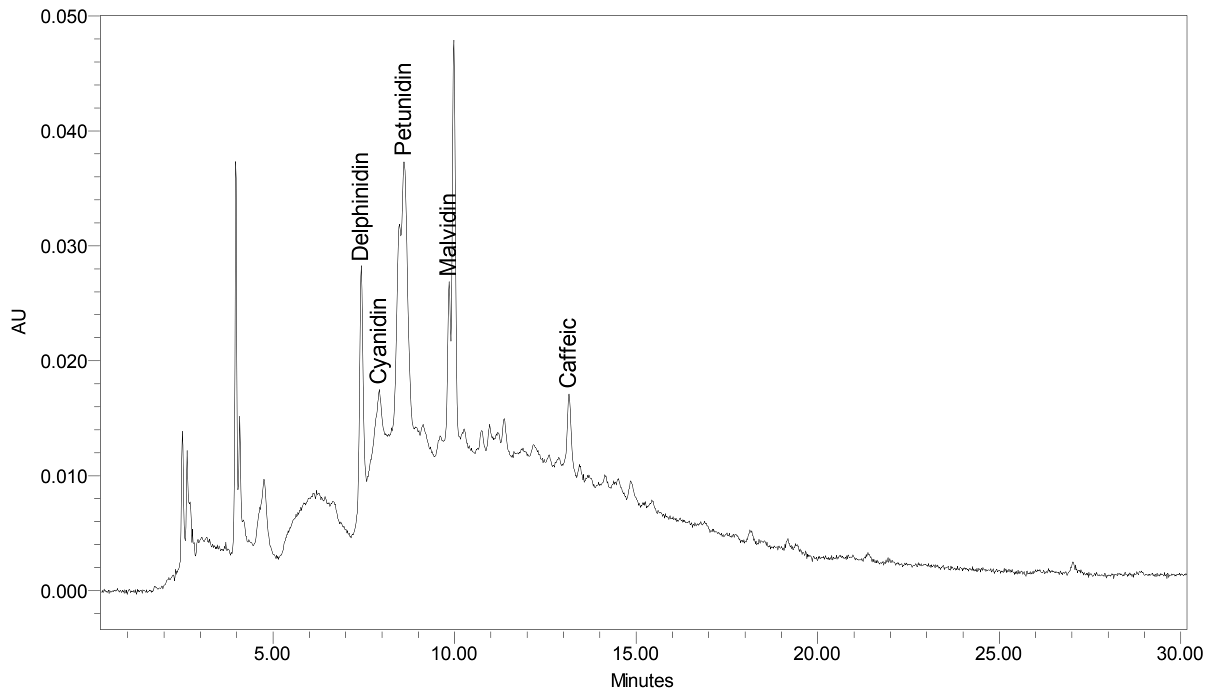
Juice at 280 nm



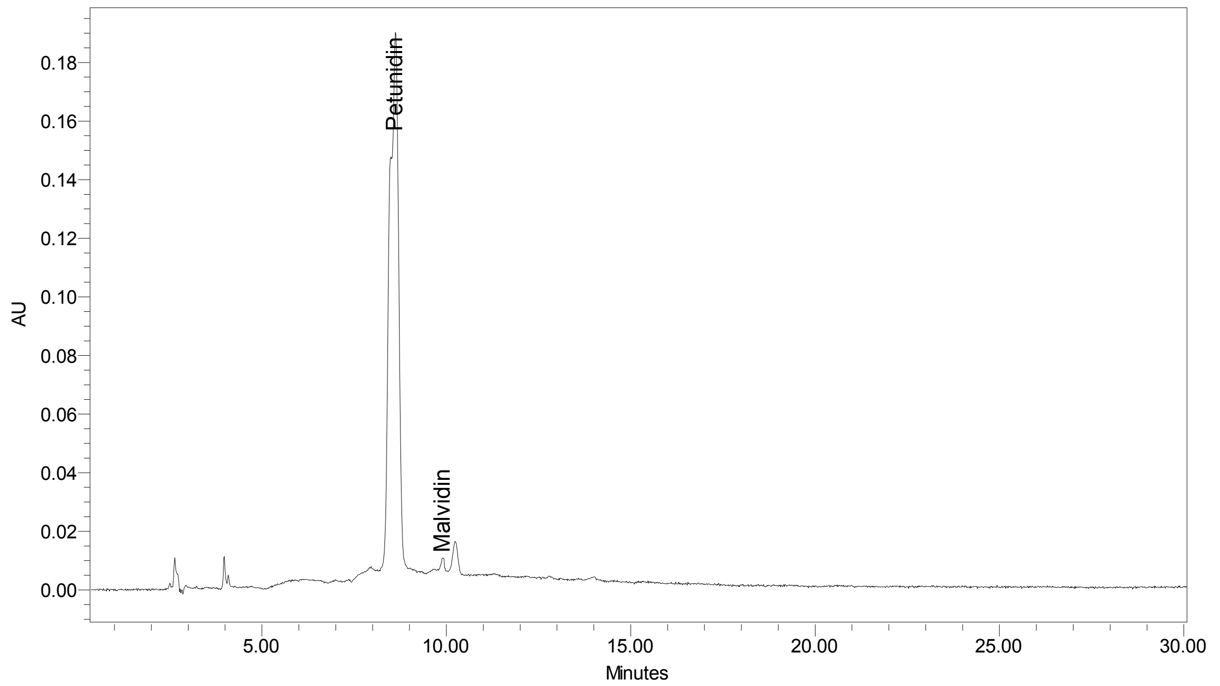
Juice at 320 nm



Juice at 370 nm

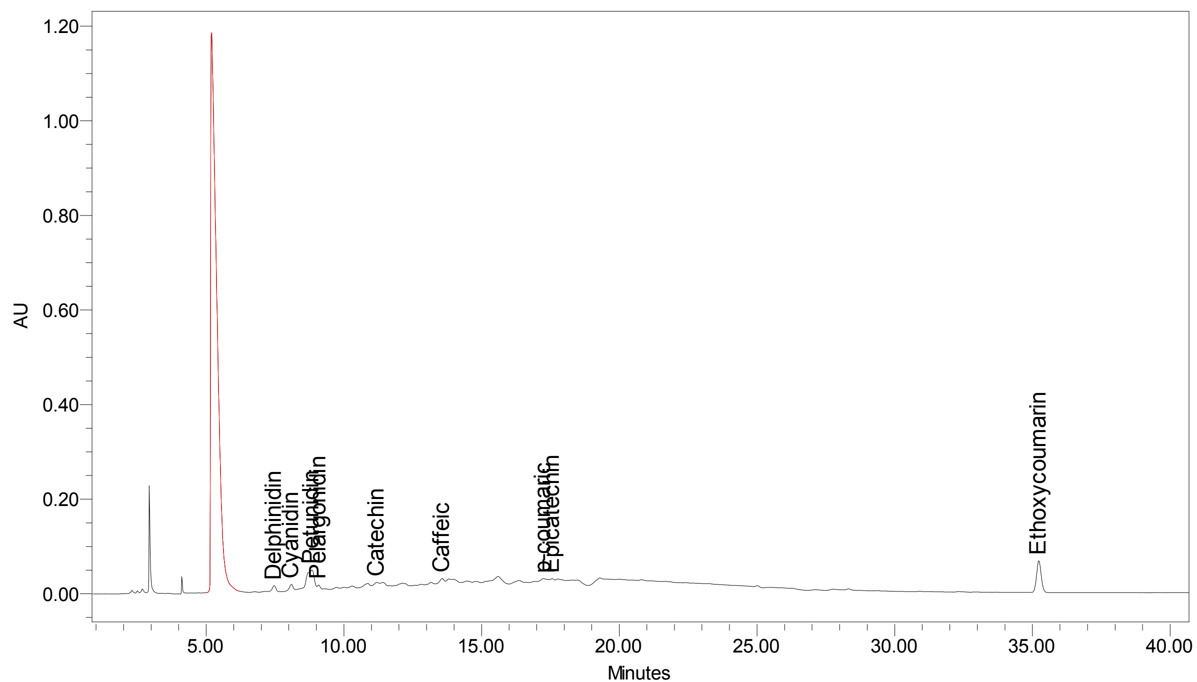


Juice at 520 nm

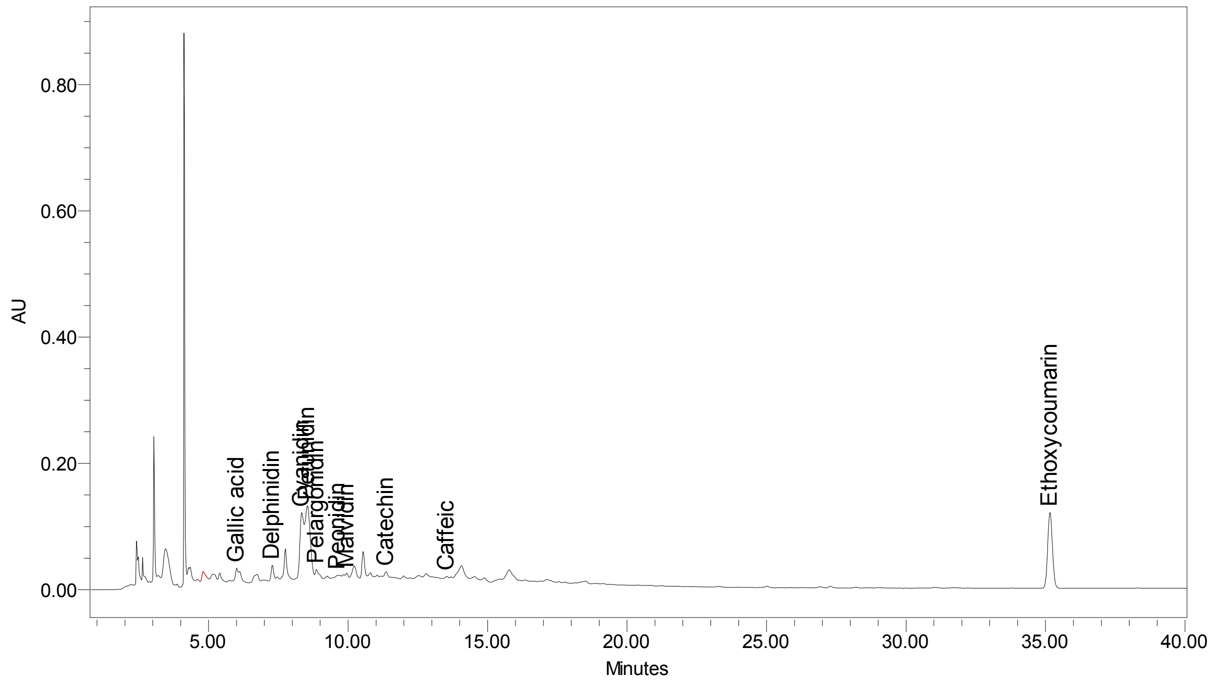


Whole grape

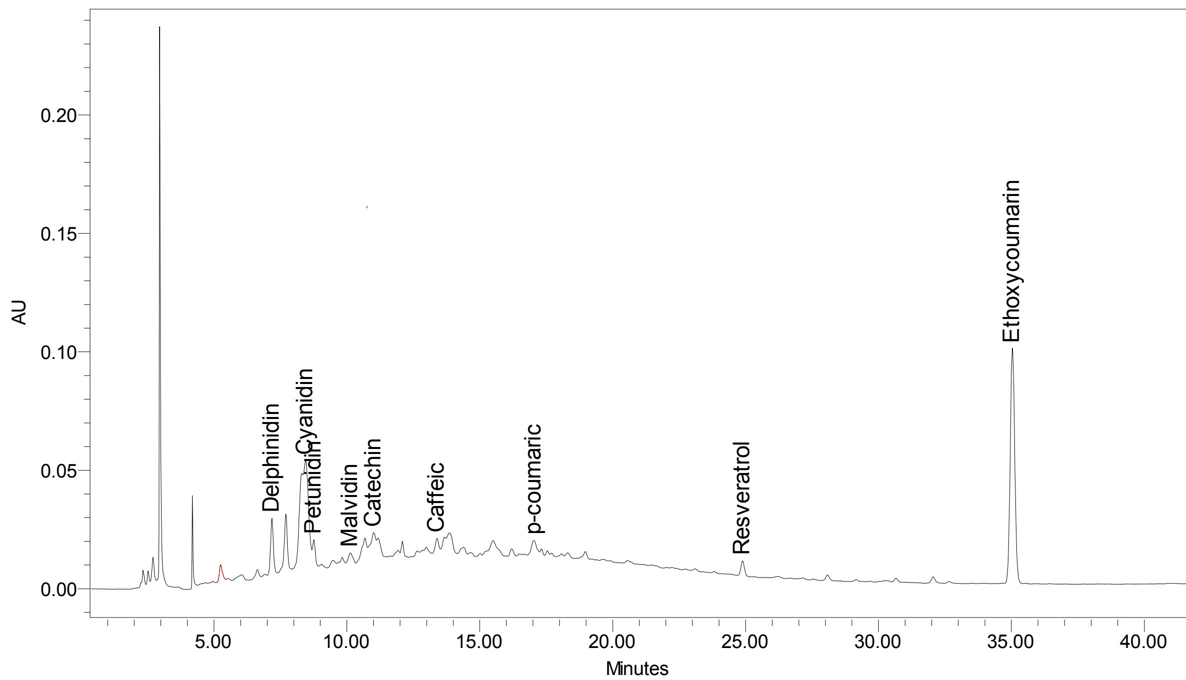
Acetone



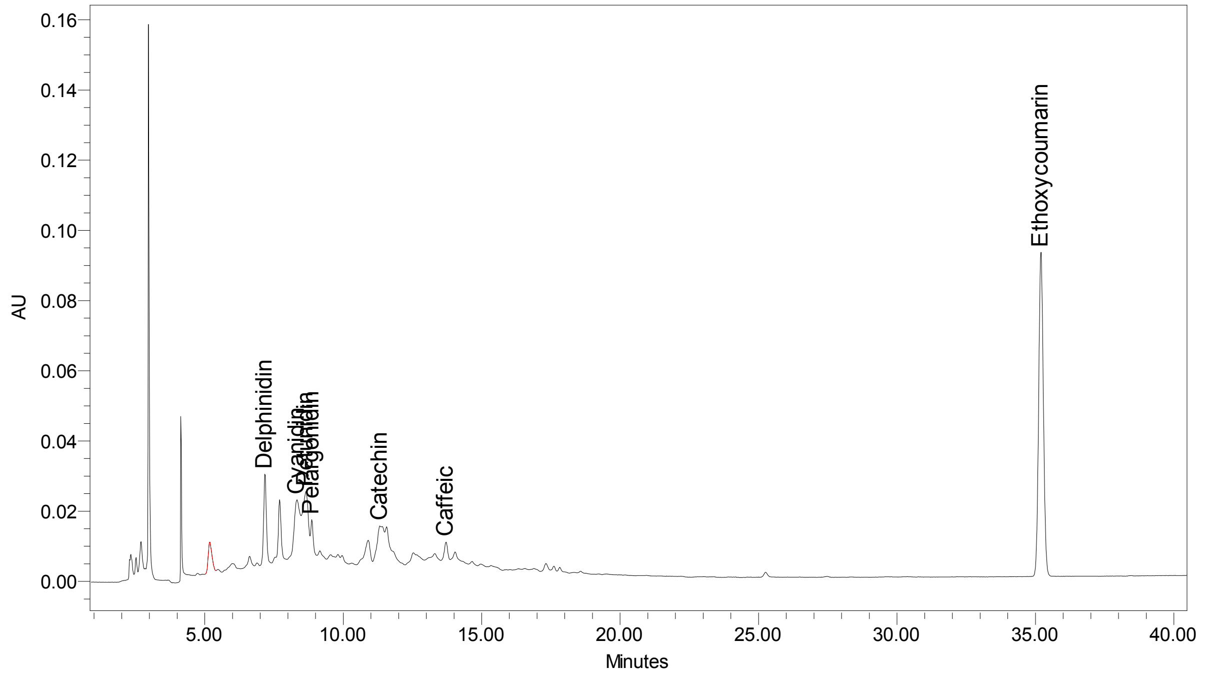
50% Methanol



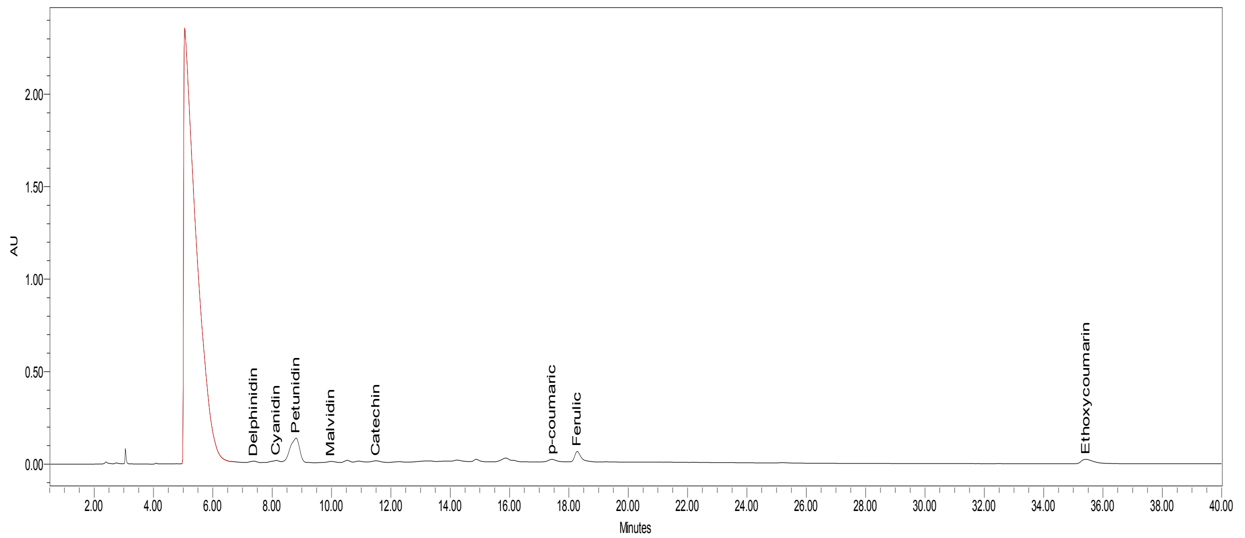
70 % Methanol



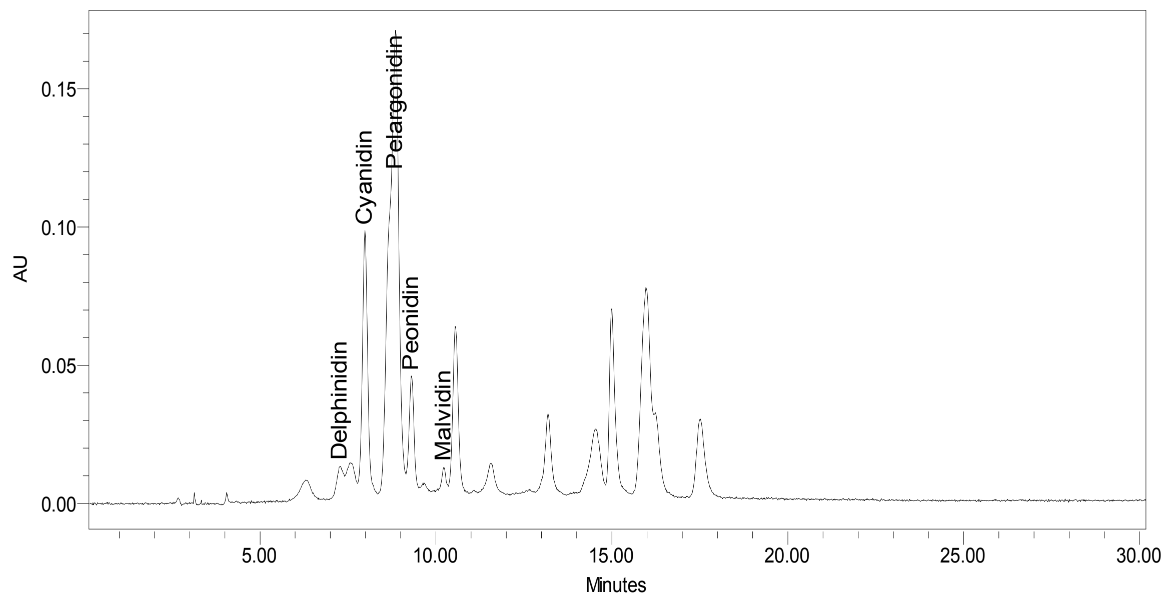
0.01% Pectinase



Petroleum Ether



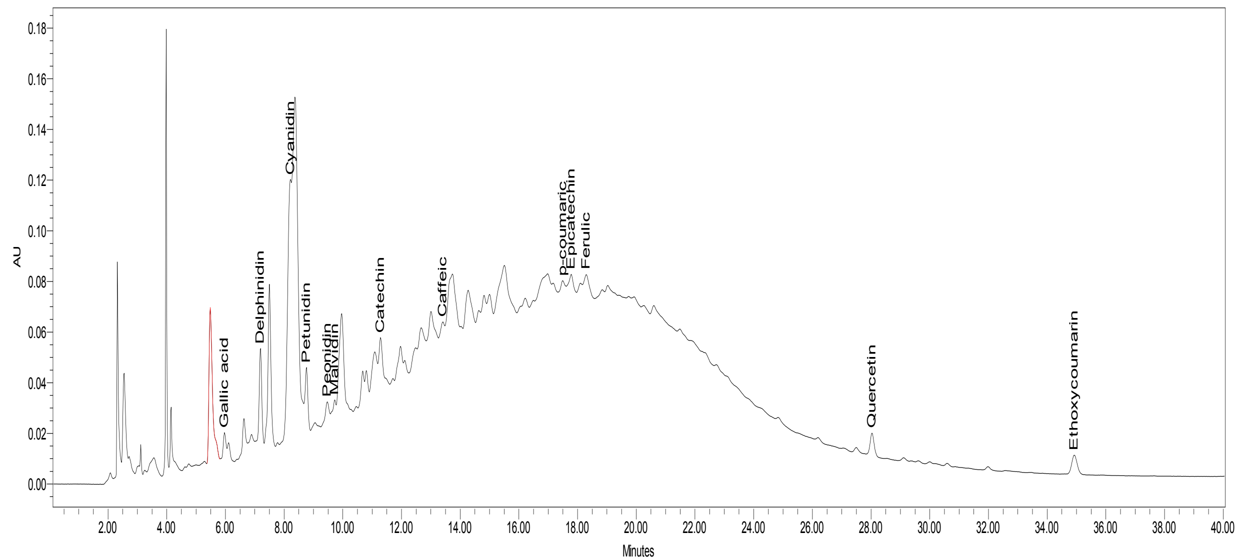
Anthocyanins identified in whole grape sample



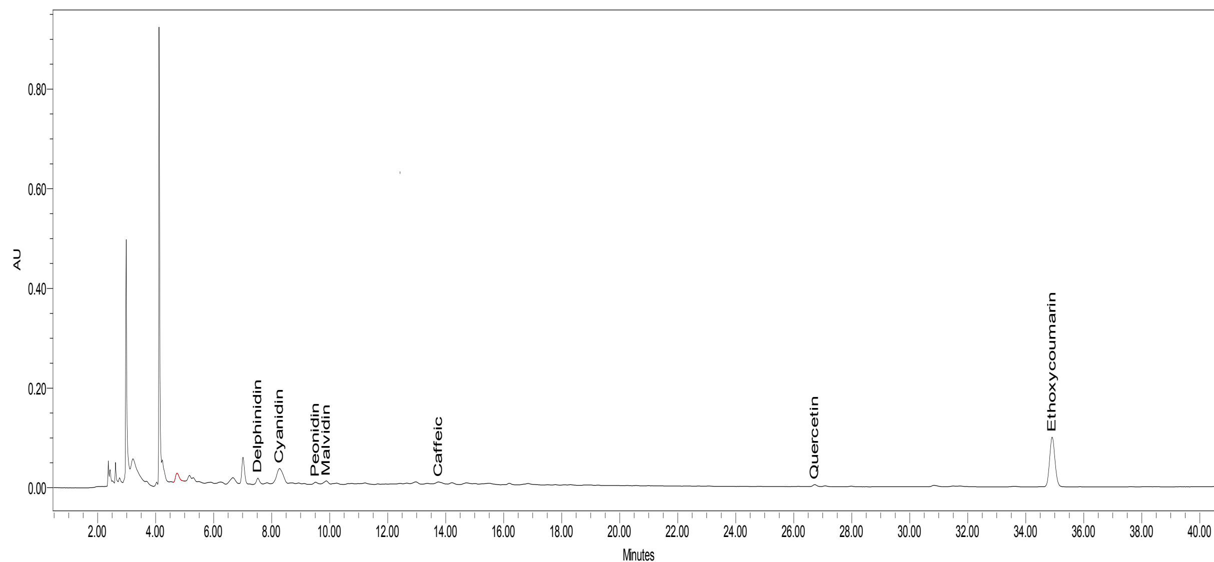
0.15
0.10
0.05
0.00
AU
Delphinidin
Cyanidin
Pelargonidin
Peonidin
Malvidin
5.00
10.00
15.00
20.00
25.00
30.00
Minutes

<u>Cynthiana pomace</u>

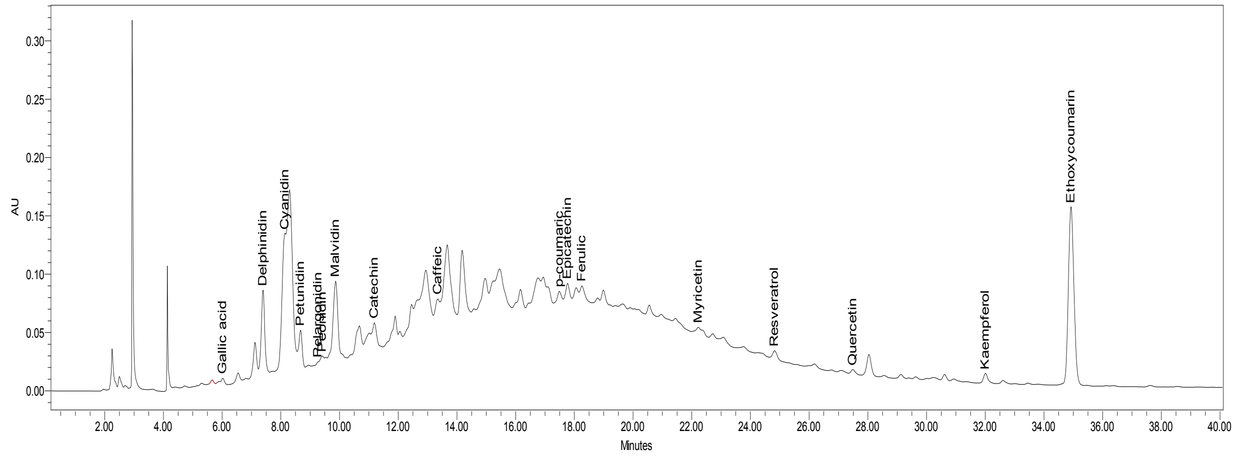
Acetone



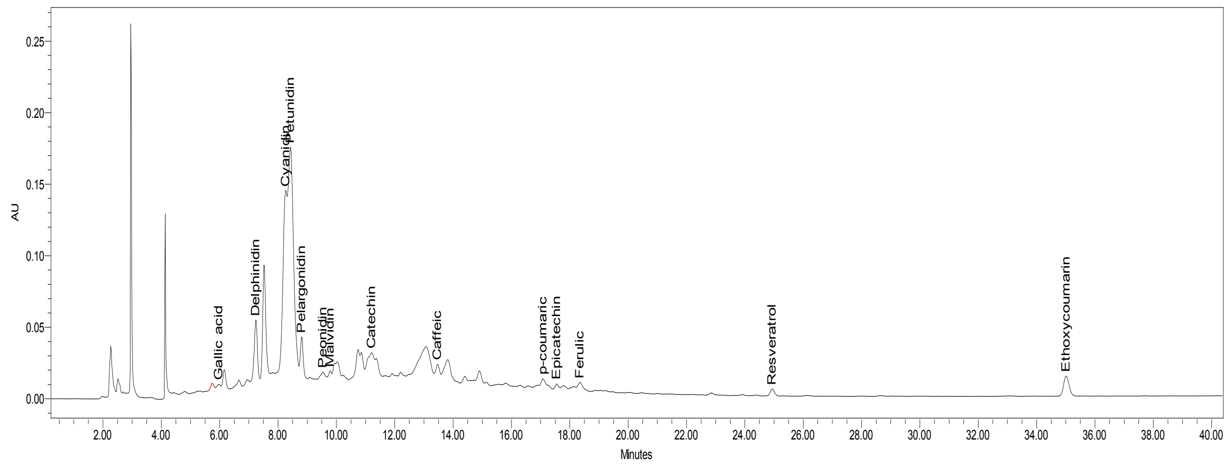
50% methanol



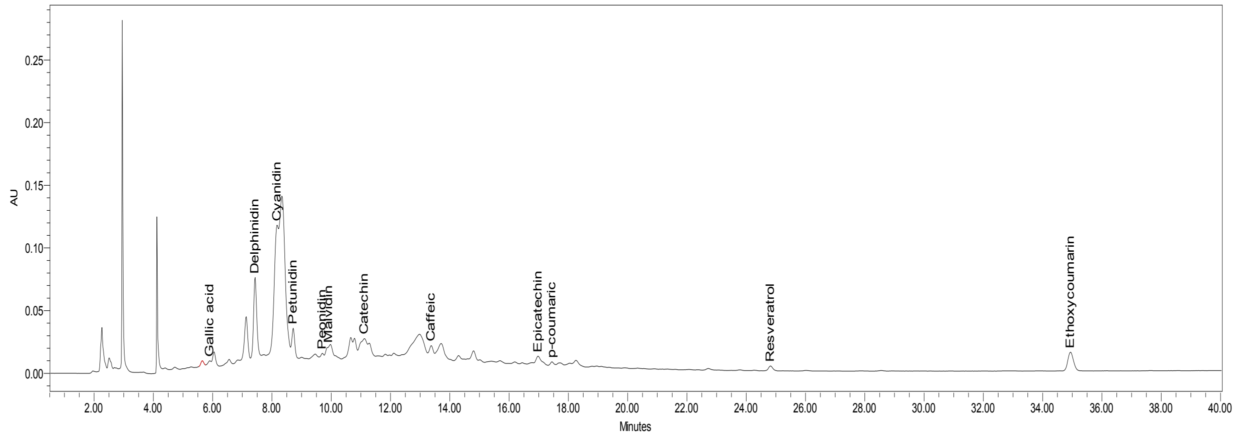
70% methanol



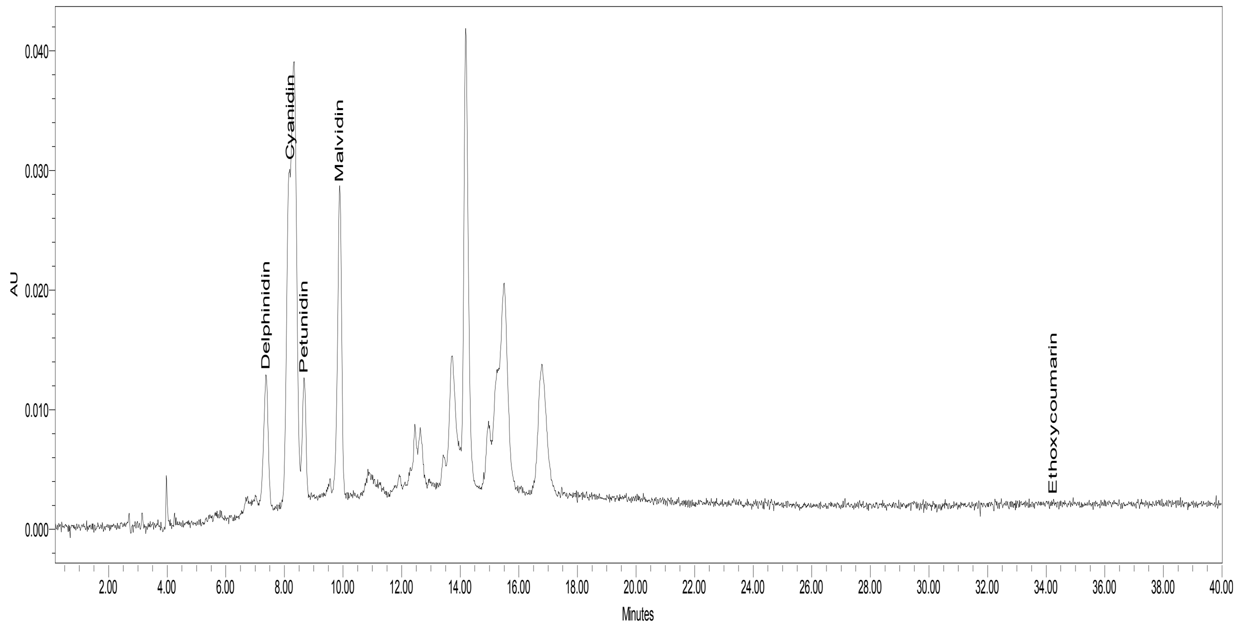
0.01% Pectinase



Petroleum ether



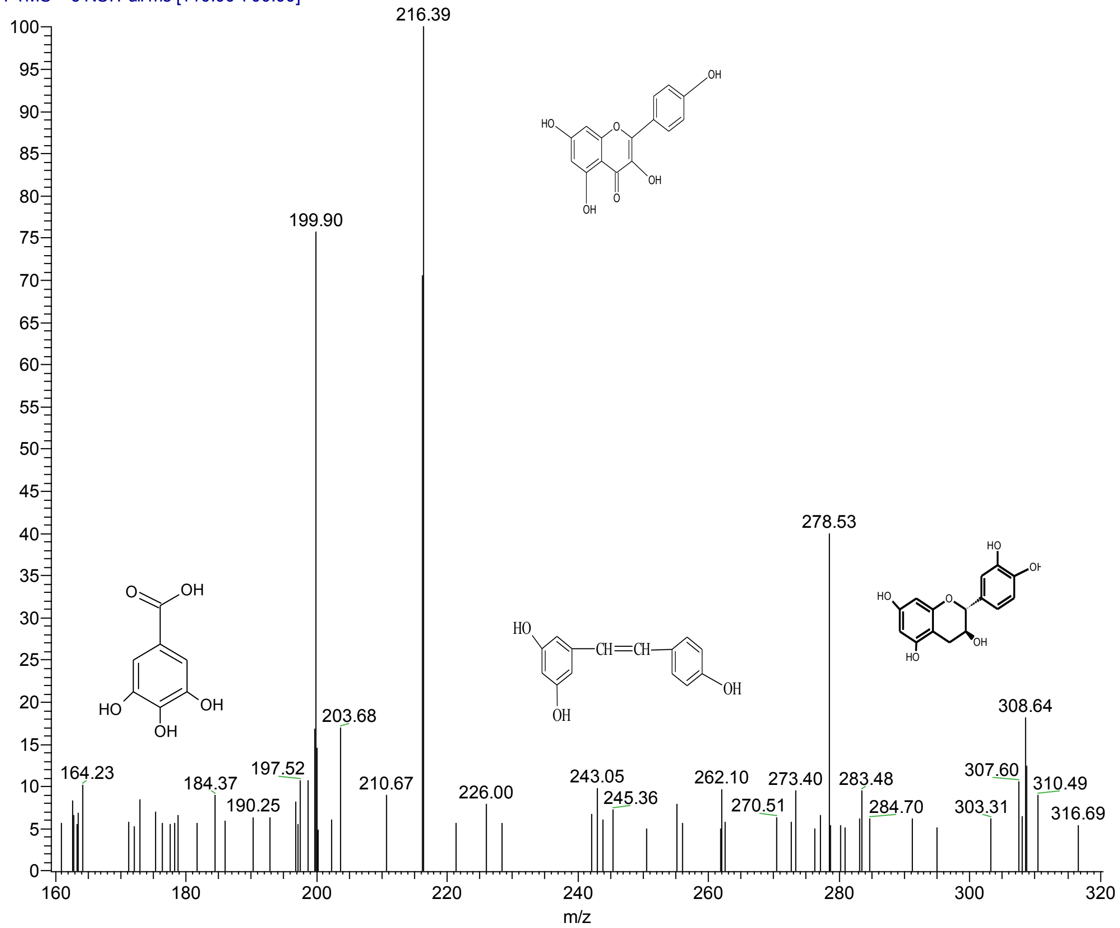
Anthocyanins identified in Cynthiana pomace



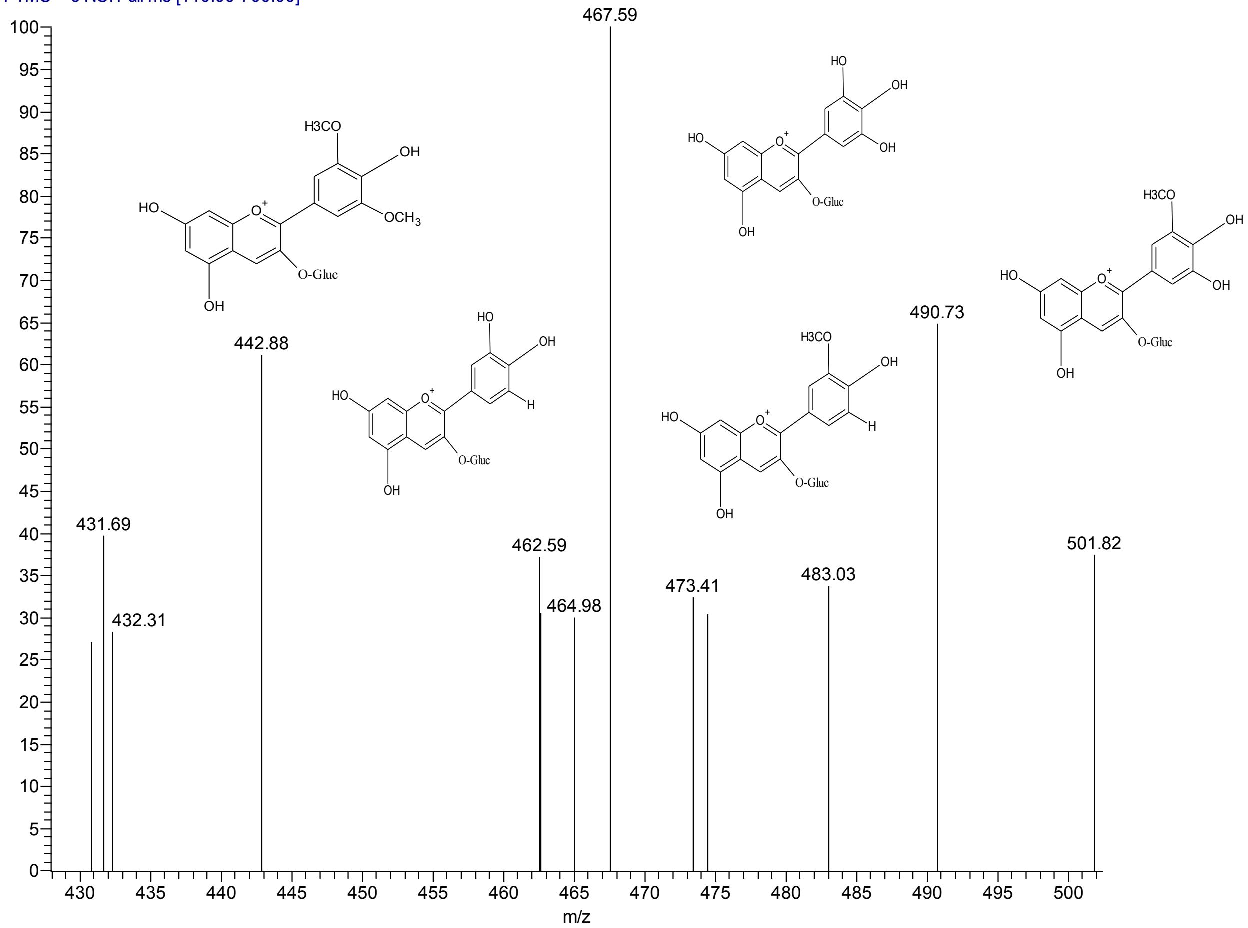
APPENDICE E

MASS SPECTROSCOPY RESULTS

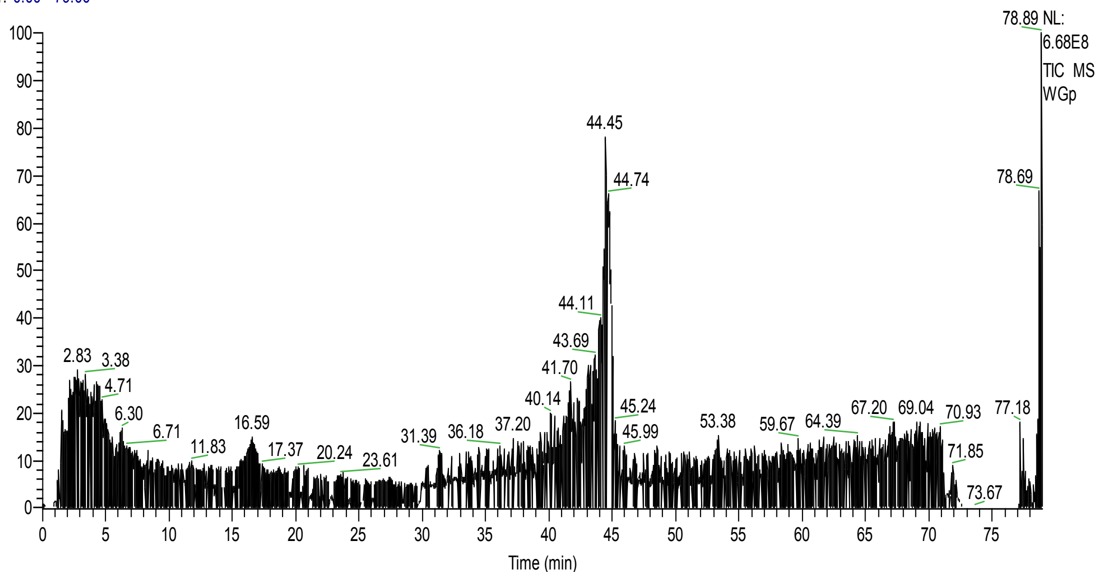
Phenolic acids, flavonols, flavonols and stilbenes standards

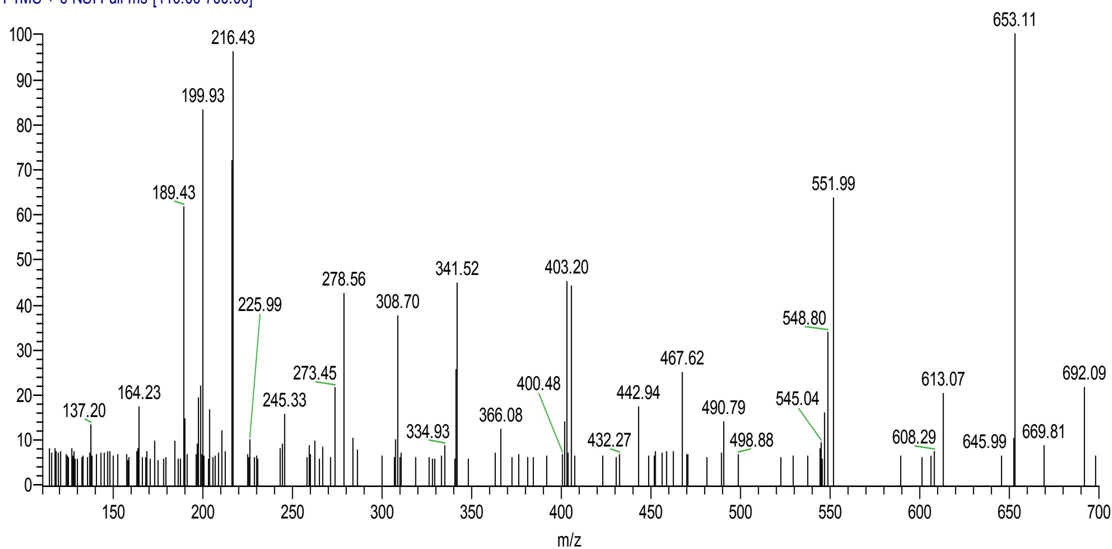


Anthocyanins Standards

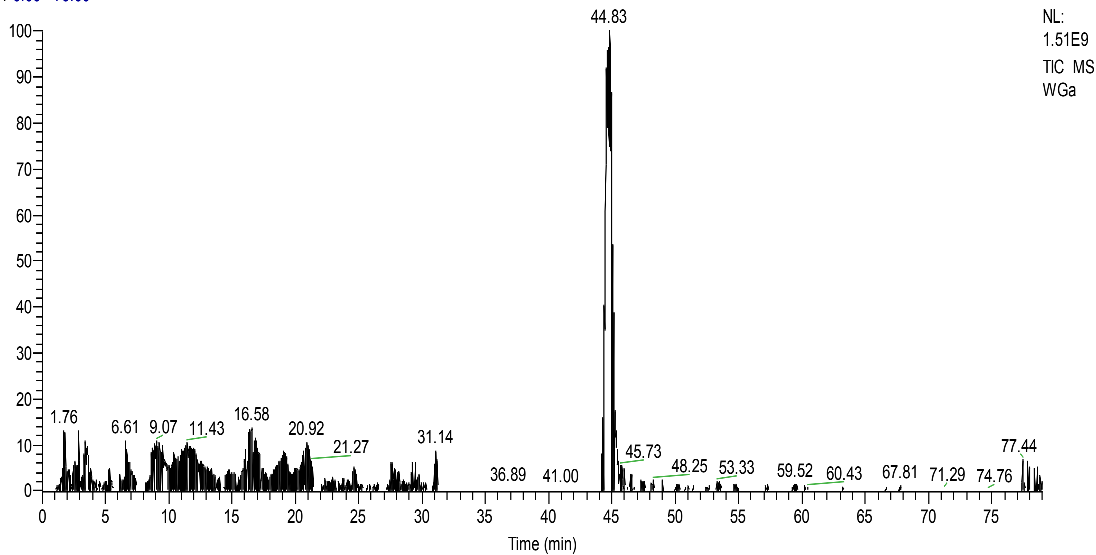


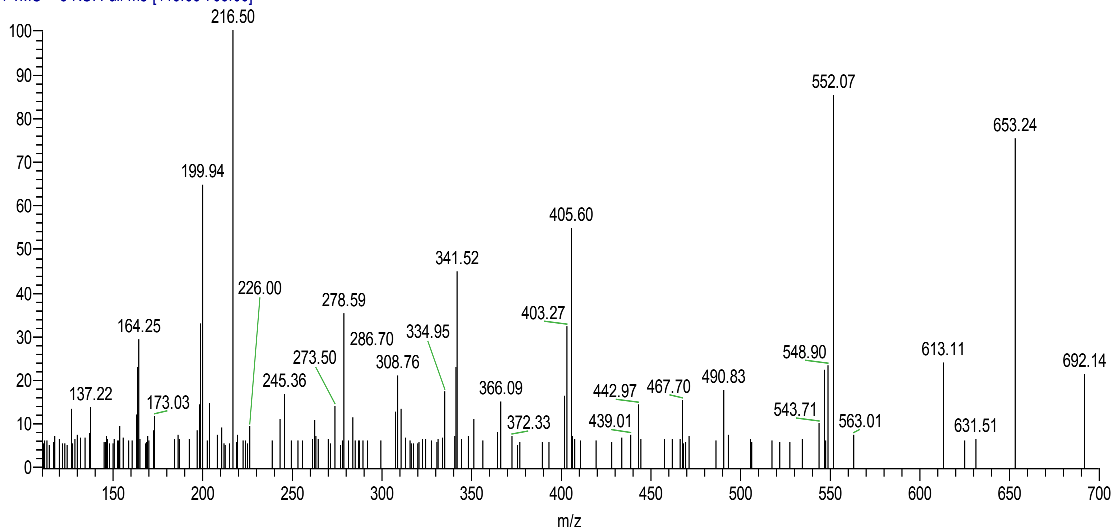
Whole grape phenolics



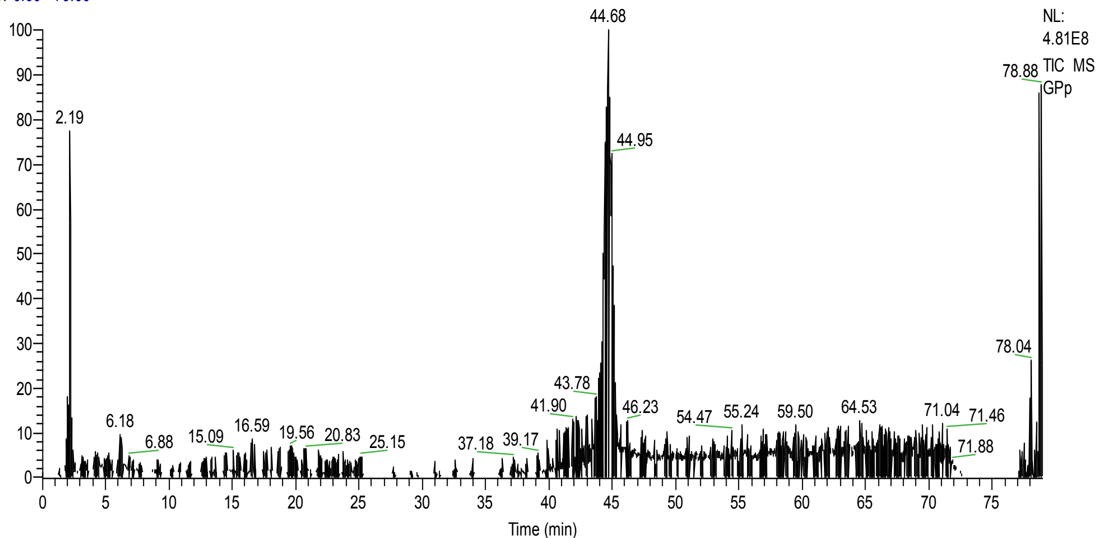


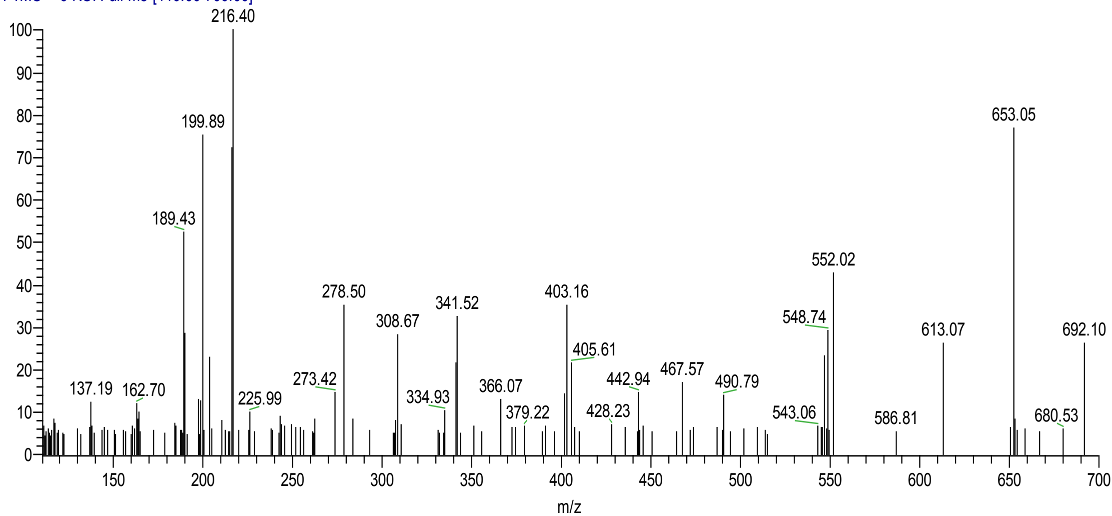
Anthocyanins





Grape pomace phenolics

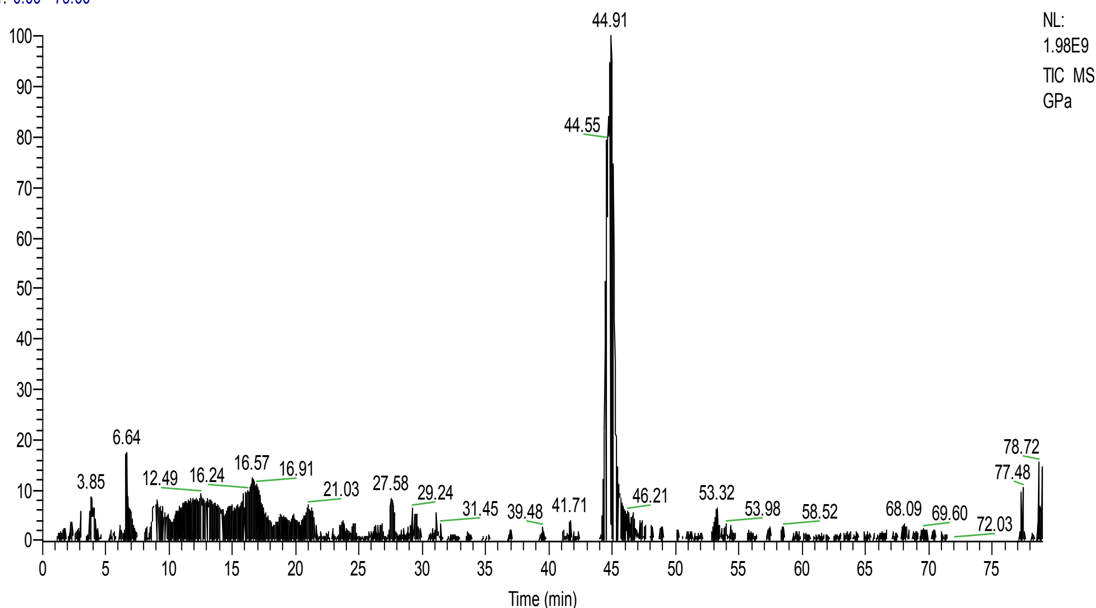




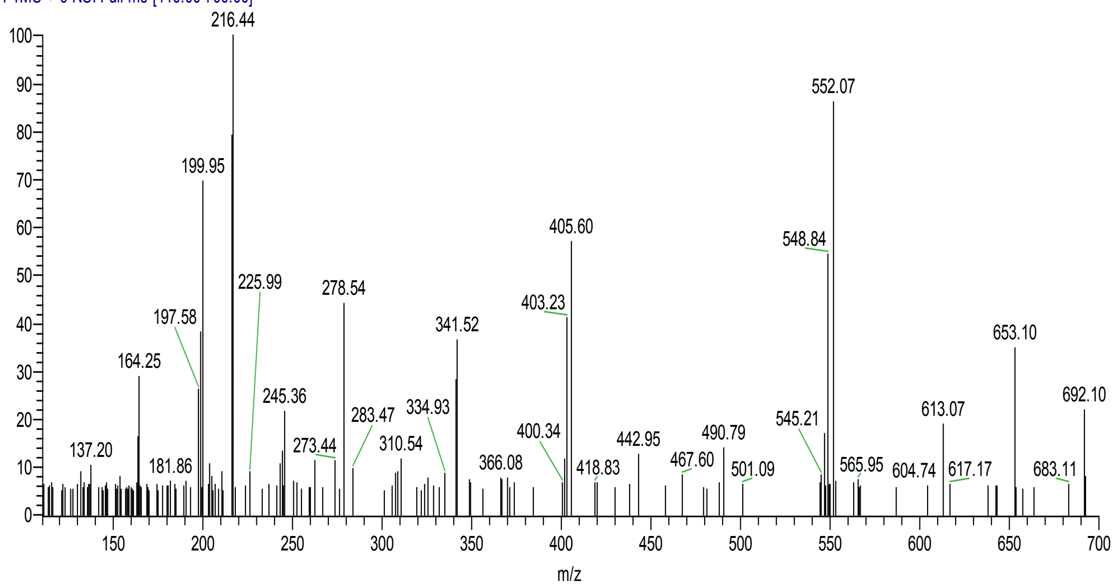
Grape pomace anthocyanins

C:\DOCUME~1\linar\LOCALS~1\Temp\GPa 1/10/2011 8:07:24 PM

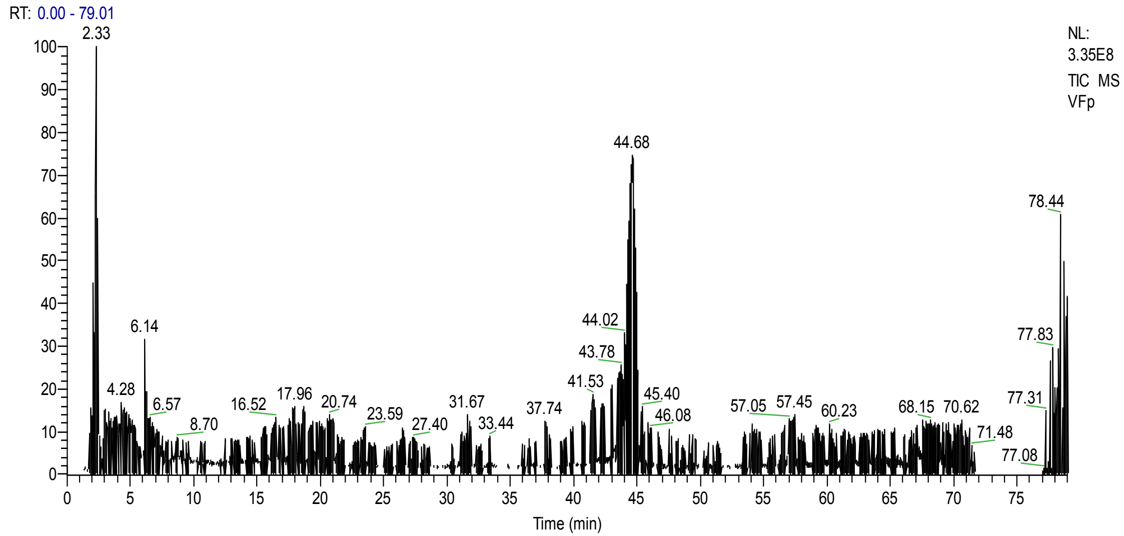
RT: 0.00 - 79.00

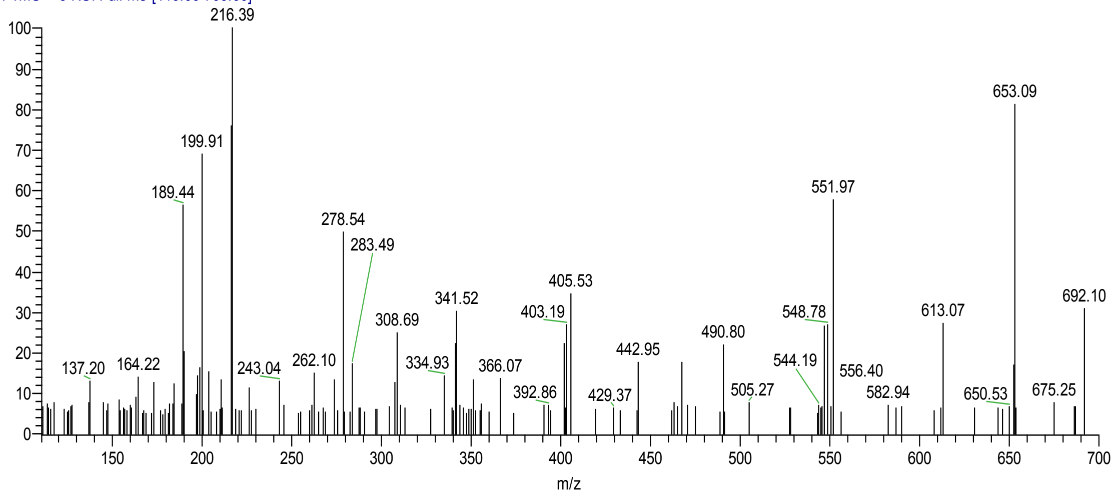


GPa #1 RT: 0.01 AV: 1 NL: 4.96E4
T: FTMS + c NSI Full ms [110.00-700.00]

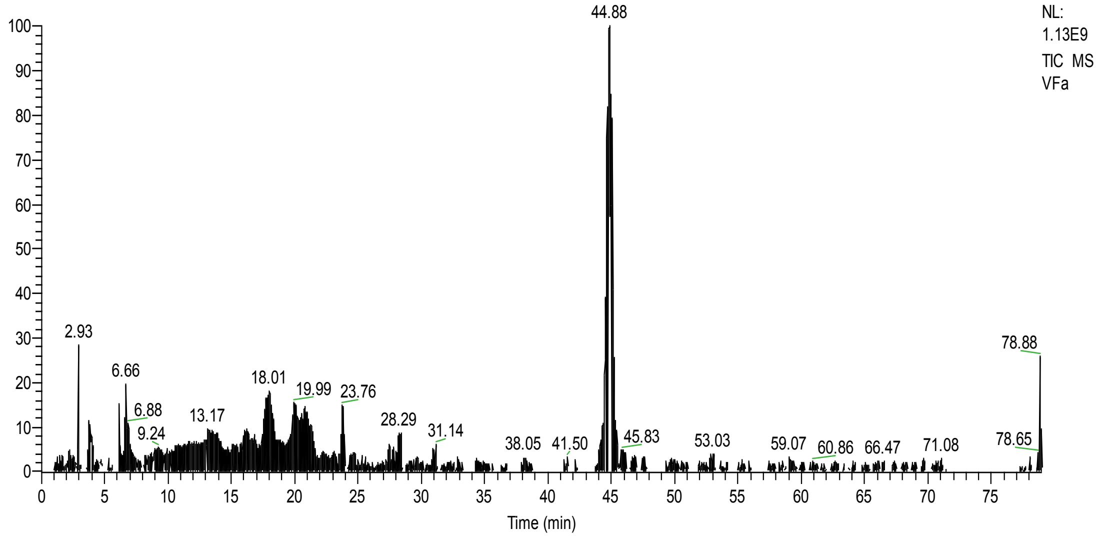


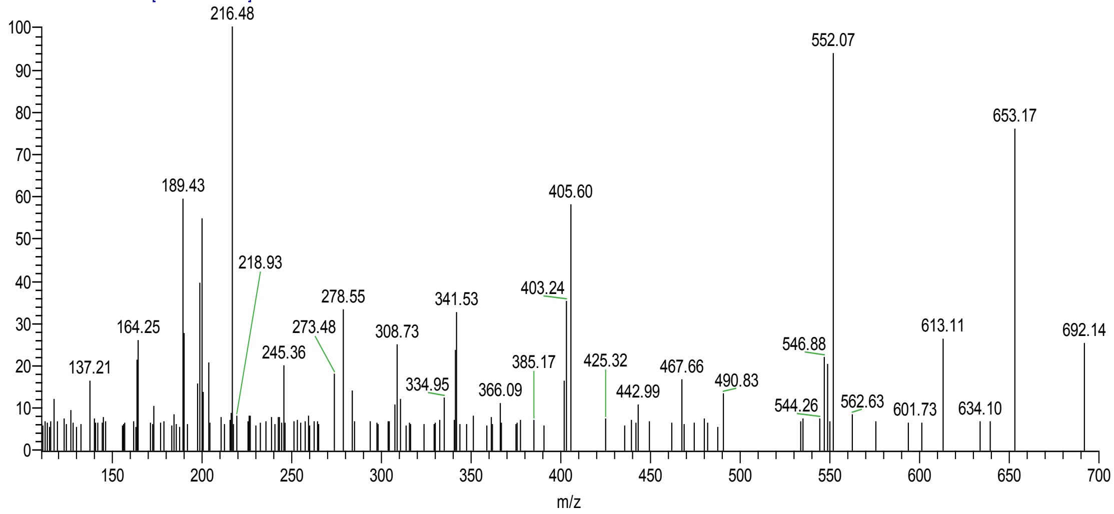
Commercial dried grape pomace Phenolics





Commercial dried grape pomace Anthocyanins





APPENDICE F

MOISTURE CONTENT OF JUICE, WHOLE GRAPE, CYNTHIANA POMACE AND COMMERCIAL DRIED GRAPE POMACE

Moisture percentage of samples

Sample	Juice	Whole grape	Cynthiana pomace	Commercial pomace
Moisture content (%)	91.46 ± 1.69	71.15 ± 0.29	46.92 ± 0.16	6.86 ± 0.07

Each value represents the mean of three replications with ± SD

APPENDICE G

IDENTIFICATION AND QUANTIFICATION OF INDIVIDUAL COMPOUNDS IN RED PIGMENTED FRACTIONS OF CYNTHIANA POMACE BY HPLC

Amberlite XAD Acetone					
Analyte	**Fraction 7**	**Fraction 8**	**Fraction 9**	**Fraction 10**	**Fraction 11**
(-) Epicatechin gallate	<0.1	2.95 ± 0.03	20.28 ± 0.01	1.13 ± 0.02	2.74 ± 0.01
(+) Catechin hydrate	88.08 ± 0.01	82.95 ± 0.02	<0.1	11.30 ± 0.05	5.17 ± 0.01
Caffeic acid	<0.1	2.18 ± 0.02	3.57 ± 0.01	1.29 ± 0.09	0.75 ± 0.01
Ferulic acid	<0.1	0.41 ± 0.03	<0.1	<0.1	1.05 ± 0.07
Gallic Acid	6.22 ± 0.01	13.50 ± 0.06	<0.1	<0.1	<0.1
Isoharmnetin	<0.1	<0.1	<0.1	<0.1	<0.1
Kaempferol	<0.1	<0.1	<0.1	<0.1	<0.1
Myricetin	<0.1	<0.1	0.22 ± 0.04	<0.1	<0.1
p-coumaric acid	<0.1	1.69 ± 0.05	2.55 ± 0.05	3.35 ± 0<0.1.03	12.73 ± 0.04
Quercetin	<0.1	<0.1	<0.1	<0.1	<0.1
Resveratrol	<0.1	<0.1	<0.1	<0.1	<0.1
Cy3G	15.89 ± 0.01	93.63 ± 0.0113	80.69 ± 0.01	11.71 ± 0.01	22.63 ± 0.01
Dp3G	17.98 ± 0.06	70.09 ± 0.01	62.23 ± 0.01	1.45 ± 0.02	8.20 ± 0.09
Mv3G	<0.1	2.22 ± 0.09	2.74 ± 0.15	6.69 ± 0.01	<0.1
Pe3G	<0.1	1.48 ± 0.07	3.35 ± 0.07	<0.1	<0.1
Pg3G	28.74 ± 0.06	70.42 ± 0.03	74.95 ± 0.01	3.24 ± 0.02	12.19 ± 0.01
Pt 3G	237.63 ± 0.06	134.52 ± 0.02	51.88 ± 1.80	<0.1	10.09 ± 0.03
TOTALS	394.55 ± 36.38	476.04 ± 13.74	302.45 ± 10.54	40.16 ± 1.55	75.55 ± 2.34

Each value represents the mean of three replications

Amberlite XAD Methanol						
Analyte	**Fraction 23**	**Fraction 24**	**Fraction 25**	**Fraction 26**	**Fraction 27**	**Fraction 28**
(-) Epicatechin gallate	3.58 ± 0.80	1.55 ± 0.15	20.28 ± 0.01	1. 88 ± 0.07	1.00 ± 0.69	2.27 ± 0.16
(+) Catechin hydrate	5.84 ± 0.3	3.11 ± 0.05	3.15 ± 0.13	8.81± 0.02	6.50 ± 0.05	3.43 ± 0.13
Caffeic acid	<0.1	<0.1	3.57 ± 0.01	<0.1	0.75 ± 0.01	<0.1
Ferulic acid	0.30 ± 0.05	<0.1	0.13 ± 0.08	<0.1	<0.1	0.48 ± 0.19
Gallic Acid	1.93 ± 0.03	1.58 ± 0.15	1.44 ± 0.03	1.61 ± 0.01	1.39 ± 0.01	1.52 ± 0.02
Isoharmnetin	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Kaempferol	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Myricetin	<0.1	<0.1	0.22 ± 0.04	<0.1	<0.1	<0.1
p-coumaric acid	<0.1	0.35 ± 0.26	0.60 ± 0.15	1.50 ± 0.32	1.96 ± 0.25	2.91 ± 0.02
Quercetin	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Resveratrol	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cy3G	8.17 ± 0.08	7.96 ± 0.03	6.34 ± 0.02	6.49 ± 0.04	4.43 ± 0.26	<0.1
Dp3G	5.04 ± 0.06	5.47 ± 1.02	4.77 ± 0.01	5.52 ± 0.03	3.17± 0.13	4.36 ± 0.11
Mv3G	<0.1	<0.1	<0.1	6.69 ± 0.01	<0.1	5.15 ± 0.05
Pe3G	<0.1	1.48 ± 0.07	3.35 ± 0.07		<0.1	<0.1
Pg3G	3.60 ± 0.02	4.05 ± 0.08	74.95 ± 0.01	4.43 ± 0.08	2.86 ± 0.09	2.92 ± 0.01
Pt 3G	10.53 ± 0.05	5.57 ± 0.02	7.59 ± 0.04	6.97 ± 0.05	5.01 ± 0.09	5.57 ± 0.06
TOTALS	39.15 ± 1.16	29.87 ± 0.88	29.31 ± 0.87	37.42 ± 0.99	26.45 ± 0.60	28.61 ± 0.55

Each value represents the mean of three replications

Ion exchange Acetone

Analyte	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6
(-) Epicatechin gallate	<0.1	0.18 ± 0.04	2.52 ± 0.06	1.13 ± 0.02	<0.1
(+) Catechin hydrate	4.53 ± 0.13	5.33 ± 0.02	7.42 ± 0.01	1.39 ± 0.09	2.33 ± 0.01
Caffeic acid	<0.1	<0.1	3.57 ± 0.01	<0.1	<0.1
Ferulic acid	<0.1	<0.1	<0.1	<0.1	<0.1
Gallic Acid	1.03 ± 0.01	1.38 ± 0.09	1.54 ± 0.01	1.10 ± 0.01	1.07 ± 0.05
Isoharmnetin	<0.1	<0.1	<0.1	<0.1	<0.1
Kaempferol	<0.1	<0.1	<0.1	<0.1	<0.1
Myricetin	<0.1	<0.1	<0.1	<0.1	<0.1
p-coumaric acid	<0.1	<0.1	<0.1	<0.1	<0.1
Quercetin	<0.1	<0.1	<0.1	<0.1	V
Resveratrol	<0.1	<0.1	<0.1	<0.1	<0.1
Cy3G	<0.1	<0.1	<0.1	<0.1	<0.1
Dp3G	<0.1	<0.1	<0.1	<0.1	<0.1
Mv3G	<0.1	<0.1	<0.1	<0.1	<0.1
Pe3G	<0.1	<0.1	<0.1	<0.1	<0.1
Pg3G	<0.1	<0.1	<0.1	<0.1	<0.1
Pt 3G	<0.1	<0.1	1.83 ± 0.05	<0.1	<0.1
TOTALS	5.58 ± 1.76	6.91 ± 1.56	13.31 ± 1.38	2.55 ± 0.41	3.40 ± 0.63

Each value represents the mean of three replications

Ion exchange Methanol

Analyte	Fraction 30	Fraction 31	Fraction 32	Fraction 33	Fraction 34
(-) Epicatechin gallate	<0.1	5.15 ± 0.71	8.47 ± 0.37	5.56 ± 0.14	5.46 ± 0.07
(+) Catechin hydrate	22.01 ± 0.05	17.77 ± 0.06	18.03 ± 0.02	13.46 ± 0.08	14.66 ± 0.02
Caffeic acid	<0.1	<0.1	<0.1	1.29 ± 0.09	<0.1
Ferulic acid	0.87 ± 0.10	0.41 ± 0.03	0.63 ± 0.08	0.20 ± 0.07	0.14 ± 0.04
Gallic Acid	2.18 ± 0.37	0.82 ± 0.51	1.53 ± 0.04	1.31 ± 0.01	1.33 ± 0.01
Isoharmnetin	<0.1	<0.1	<0.1	<0.1	<0.1
Kaempferol	<0.1	<0.1<0.1	<0.1	<0.1	<0.1
Myricetin	<0.1	3.23 ± 0.03	<0.1	<0.1	<0.1
p-coumaric acid	5.01 ± 0.15	2.36 ± 1.19	4.19 ± 0.08	1.52 ± 1.14	1.74 ± 1.26
Quercetin	<0.1	<0.1	<0.1	<0.1	<0.1
Resveratrol	<0.1	<0.1	<0.1	<0.1	<0.1
Cy3G	4.26 ± 0.02	2.74 ± 0.01	3.45 ± 0.08	1.75 ± 0.15	1.75 ± 0.10
Dp3G	<0.1	<0.1	<0.1	<0.1	<0.1
Mv3G	0.52 ± 0.16	<0.1	<0.1	<0.1	<0.1
Pe3G	<0.1	<0.1	3.35 ± 0.07	<0.1	<0.1
Pg3G	7.64 ± 0.05	5.08 ± 0.06	6.33 ± 0.01	3.34 ± 0.02	2.98 ± 0.13
Pt 3G	26.34 ± 0.03	18.51 ± 0.05	21.49 ± 0.05	14.00 ± 0.04	13.76 ± 0.01
TOTALS	81.30 ± 3.13	57..73 ± 2.12	64.39 ± 2.40	41.13 ± 1.96	41.83 ± 2.04

Each value represents the mean of three replications

APPENDICE H

ABSORBANCE OF THE NON-PIGMENTED FRACTIONS

Amberlite-Acetone				
Fraction/wv	280 nm	320 nm	370 nm	520 nm
1	0.94	0.29	0.5	0.06
2	0.63	0.44	0.87	0.05
3	0.25	0.68	0.52	0.03
4	0.48	0.50	0.33	0.07
5	0.55	0.98	0.26	0.05
6	0.98	0.03	0.58	0.3
12	0.33	0.8	0.2	0.04
13	0.02	0.06	0.03	0.01

Each value represents the mean of three replications

Amberlite-Methanol				
Fraction /wv	280 nm	320	370	520
1	0.01	0.01	0.01	0.01
2	0.01	0.01	0.01	0.01
3	0.01	0.01	0.01	0.01
4	0.01	0.01	0.01	0.01
5	0.01	0.01	0.01	0.01
6	0.01	0.01	0.01	0.01
7	0.01	0.01	0.01	0.01
8	0.01	0.01	0.01	0.01
9	0.01	0.01	0.01	0.01
10	0.01	0.01	0.01	0.01
11	0.01	0.01	0.01	0.01
12	0.01	0.01	0.01	0.01
13	0.01	0.01	0.01	0.01
14	0.01	0.01	0.01	0.01
15	0.01	0.01	0.01	0.01
16	0.01	0.01	0.01	0.01
17	0.01	0.01	0.01	0.01
18	0.32	0.01	0.01	0.01
19	0.47	0.01	0.01	0.01
20	0.55	0.01	0.01	0.01
21	0.53	0.01	0.01	0.01
22	0.94	0.96	0.62	0.01
29	0.84	0.8	0.3	0.01
30	0.76	0.5	0.2	0.01

Each value represents the mean of three replications

Iox Exchange-Acetone

Iox Exchange Acetone				
Fraction/Wv	**280 nm**	**320 nm**	**370 nm**	**520 nm**
1	0.07	0.04	0.09	0.01
7	0.20	0.53	0.07	0.01
8	0.01	0.01	0.01	0.01

Each value represents the mean of three replications

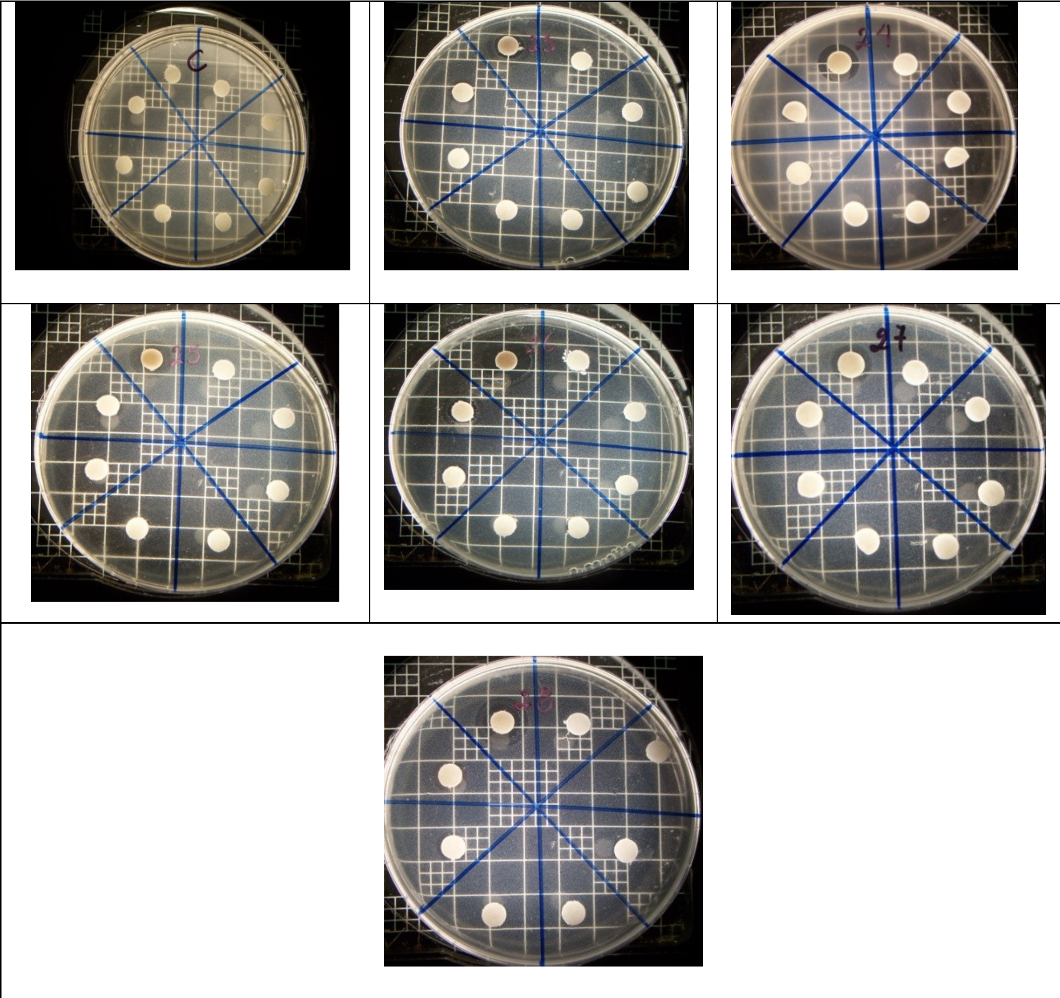
Ion Exchange Methanol				
Fraction/Wv	**280 nm**	**320 nm**	**370 nm**	**520 nm**
1	0.01	0.01	0.01	0.01
2	0.01	0.01	0.01	0.01
3	0.01	0.01	0.01	0.01
4	0.01	0.01	0.01	0.01
5	0.01	0.01	0.01	0.01
6	0.01	0.01	0.01	0.01
7	0.01	0.01	0.01	0.01
8	0.01	0.01	0.01	0.01
9	0.01	0.01	0.01	0.01
10	0.01	0.01	0.01	0.01
11	0.01	0.01	0.01	0.01
12	0.01	0.01	0.01	0.01
13	0.01	0.01	0.01	0.01
14	0.01	0.01	0.01	0.01
15	0.01	0.01	0.01	0.01
16	0.01	0.01	0.01	0.01
17	0.01	0.01	0.01	0.01
18	0.01	0.01	0.01	0.01
19	0.01	0.01	0.01	0.01
20	0.01	0.01	0.01	0.01
21	0.01	0.01	0.01	0.01
22	0.01	0.01	0.01	0.01
23	0.01	0.01	0.01	0.01
24	0.01	0.01	0.01	0.01
25	0.03	0.01	0.01	0.01
26	0.05	0.01	0.01	0.01
27	0.10	0.02	0.01	0.02
28	0.70	0.16	0.02	0.05
29	0.83	0.33	0.14	0.24
35	0.91	0.26	0.23	0.11
36	0.04	0.01	0.05	0.06
37	0.03	0.02	0.06	0.02

Each value represents the mean of three replications

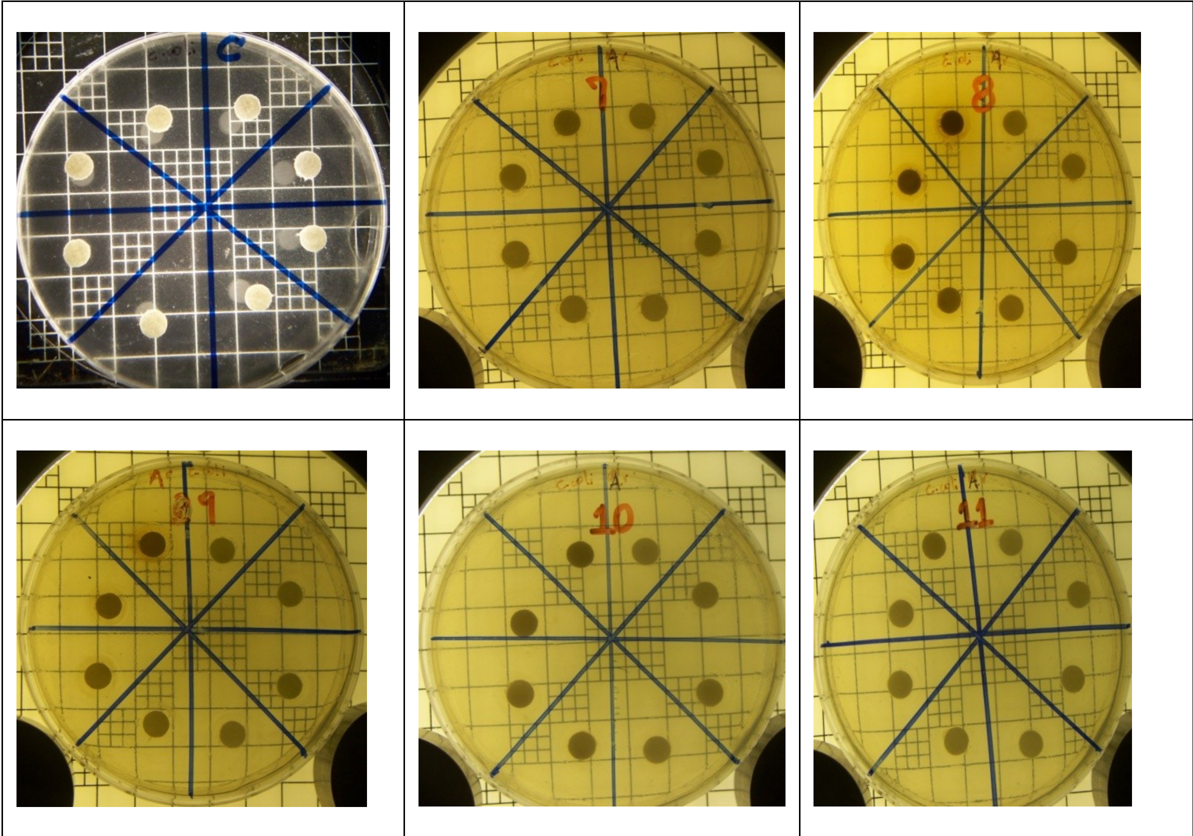
APPENDICE I

MICROBIAL STUDY ON CYNTHIANA POMACE

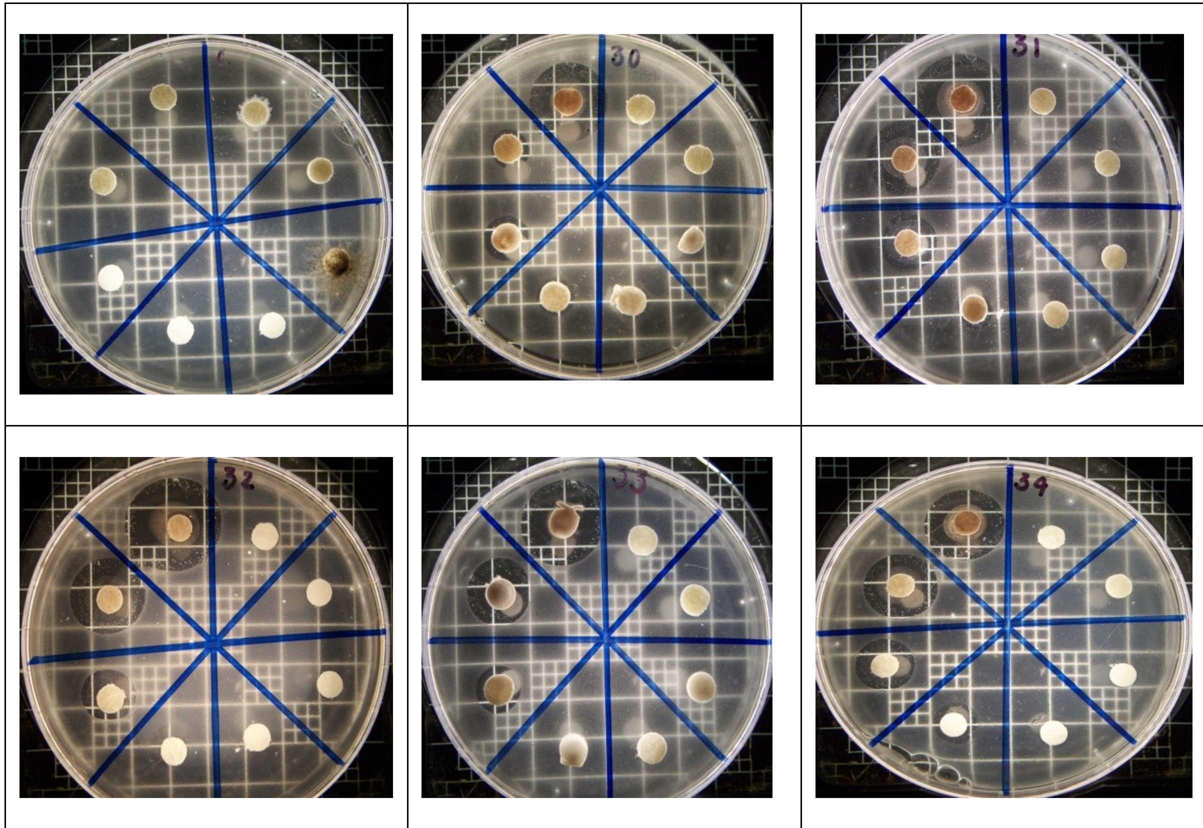
Ambelite-Methanol *E.coli*



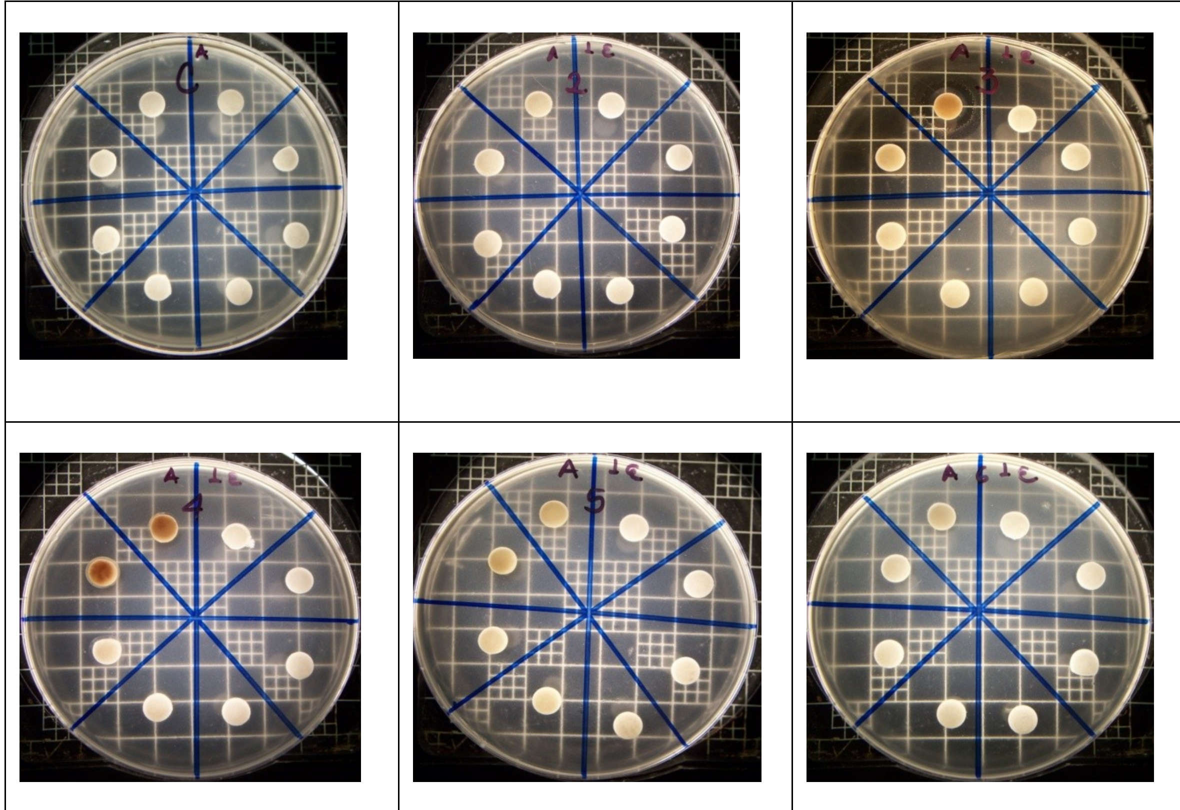
Amberlite-Acetone *E.coli*



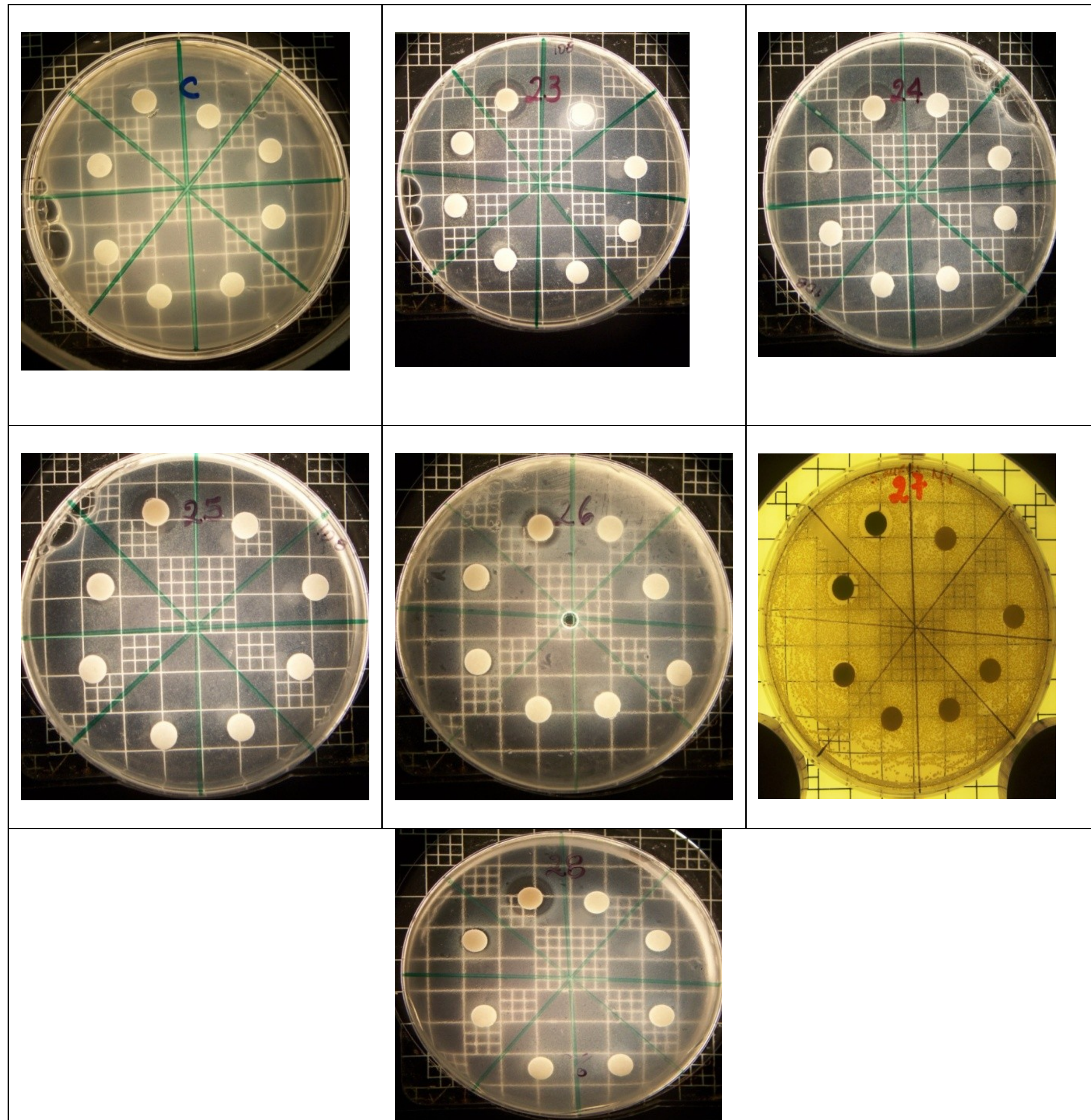
Ion Exchange-Methanol *E.coli*



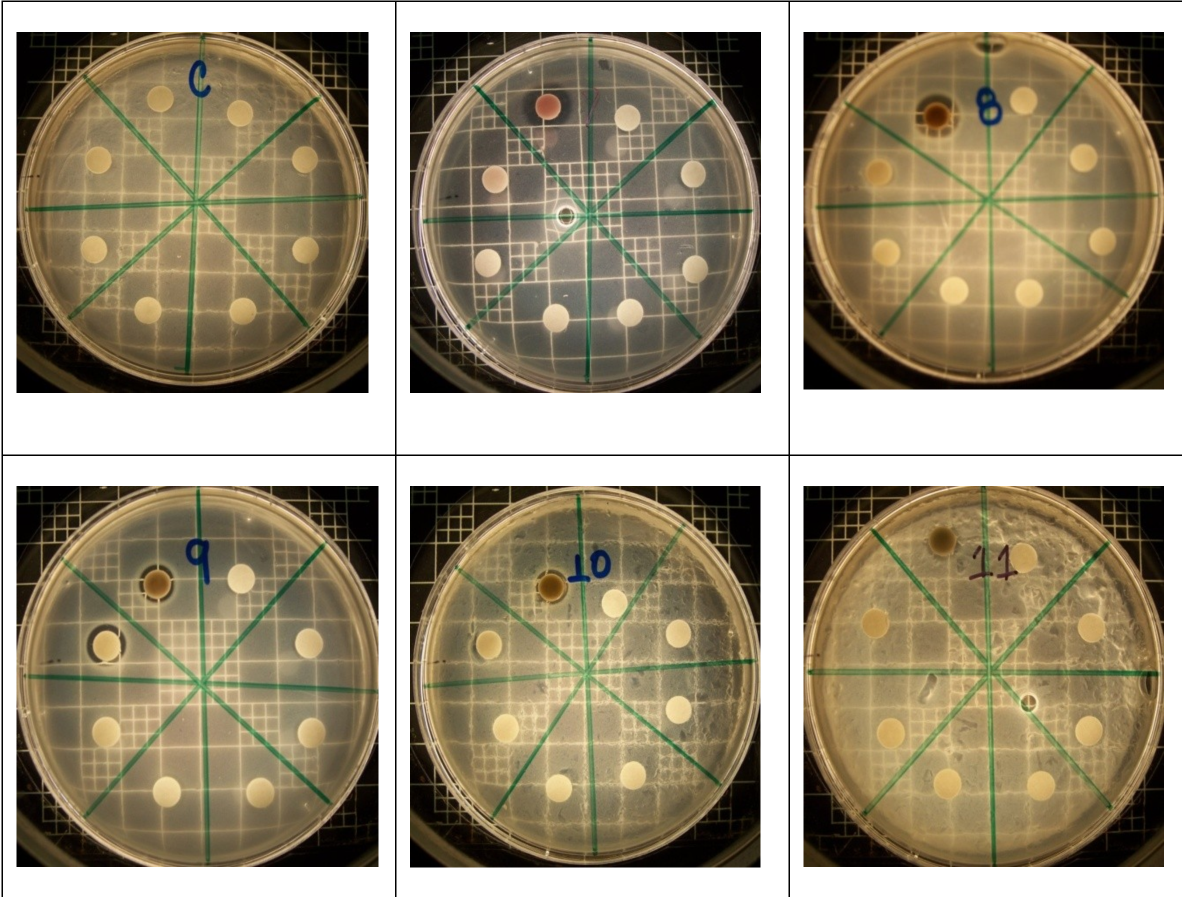
Ion exchange- Acetone *E.coli*



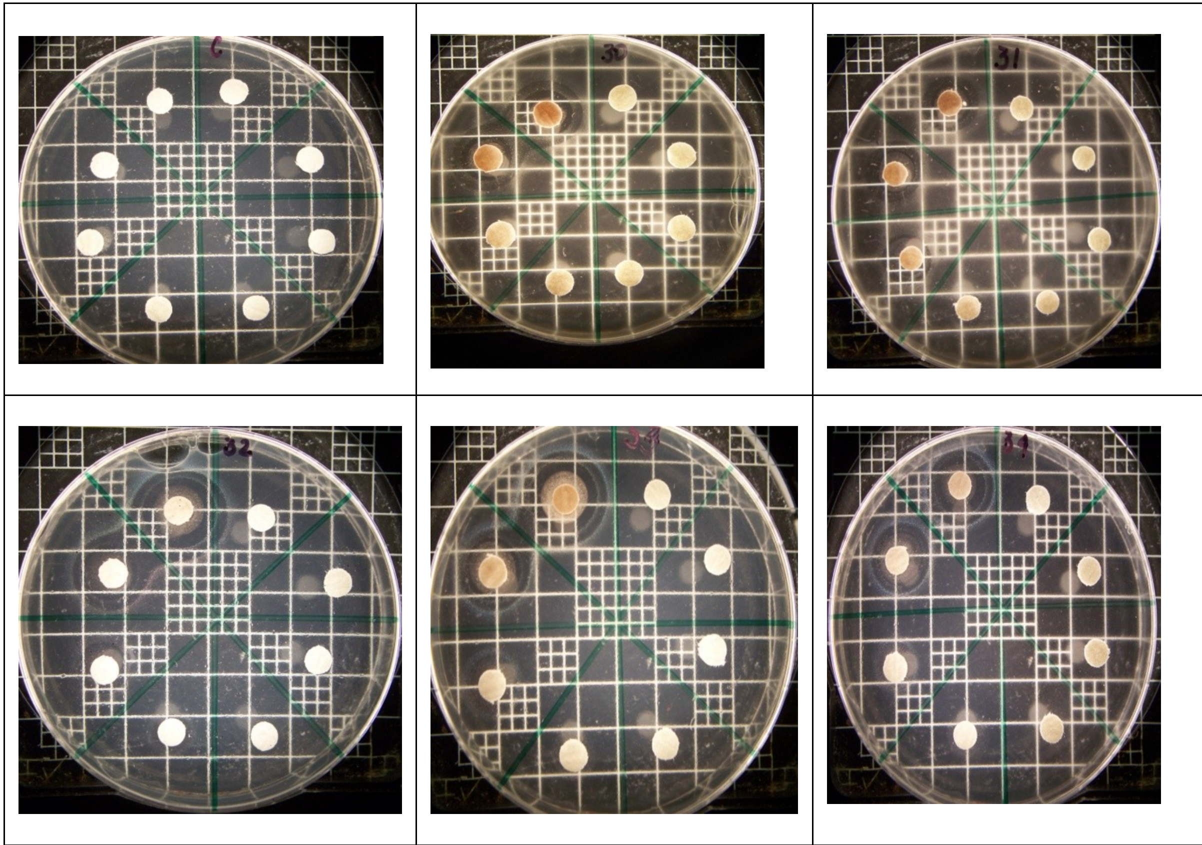
Amberlite-Methanol *S. aureus*



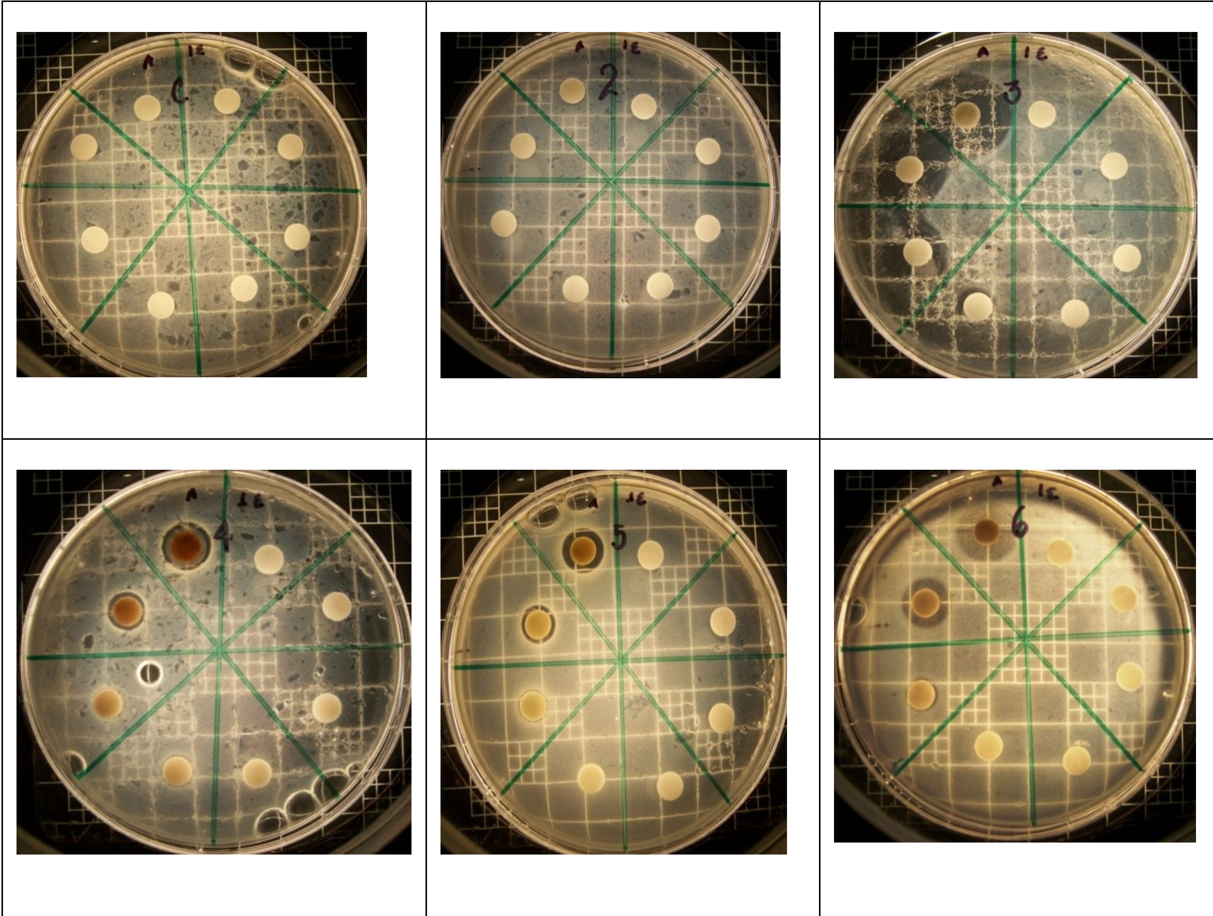
Amberlite- Acetone *S. aureus*



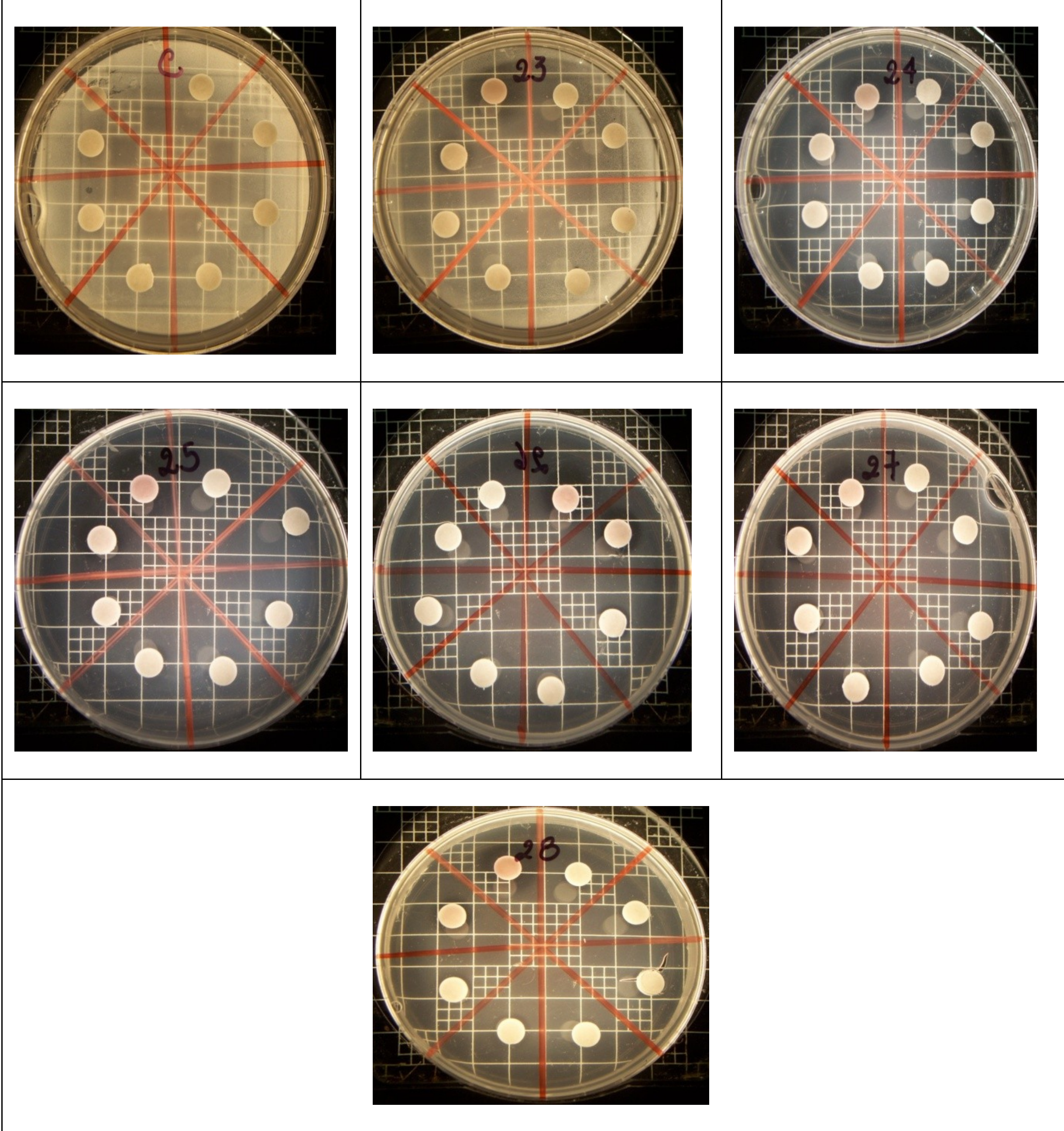
Ion exchange-Methanol *S. aureus*



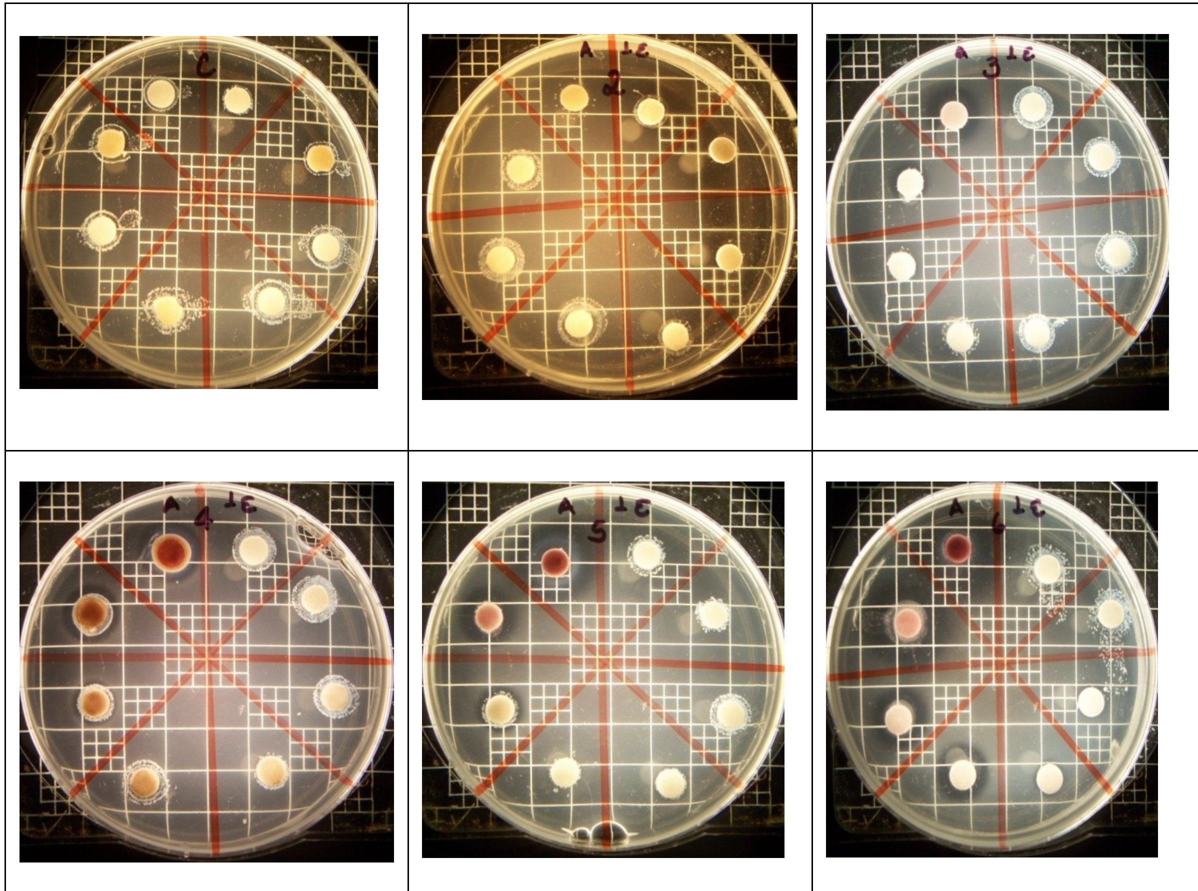
Ion Exchange Acetone *S. aureus*



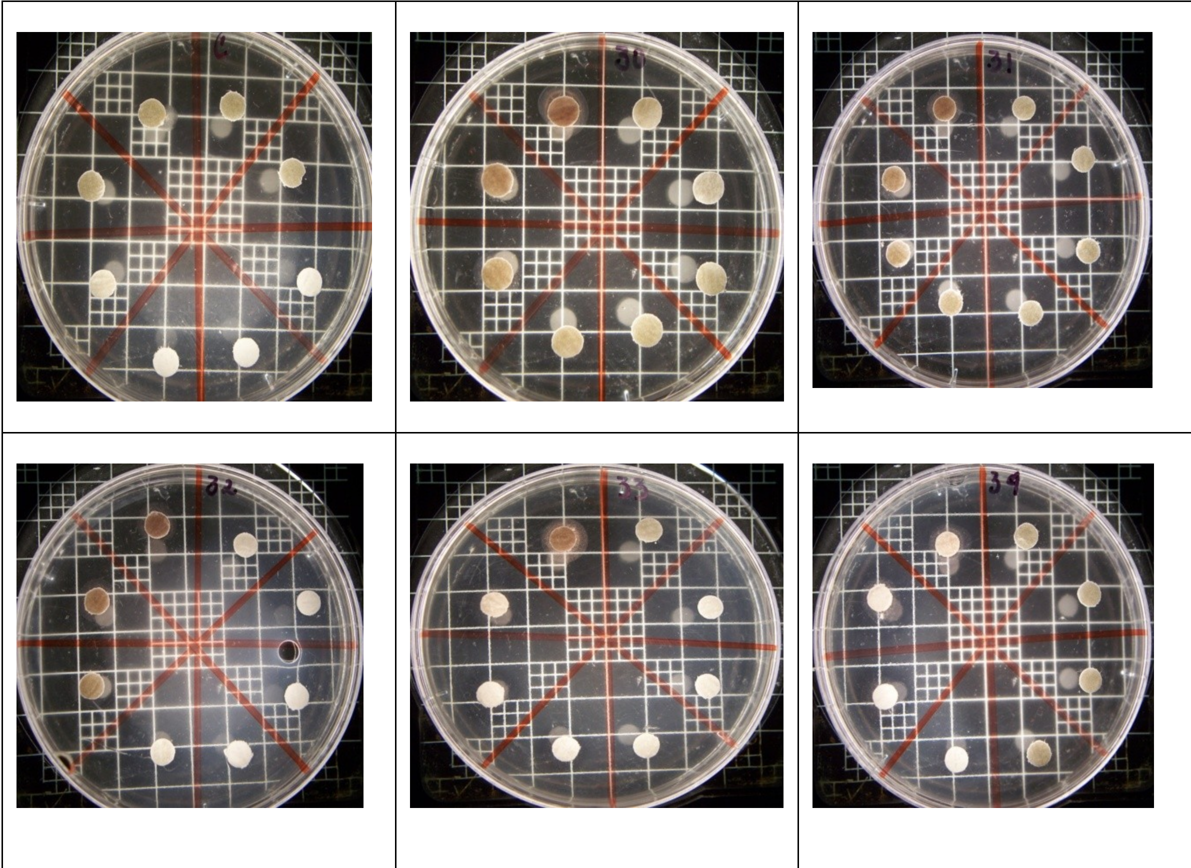
Amberlite Methanol *L.monocytogenes*



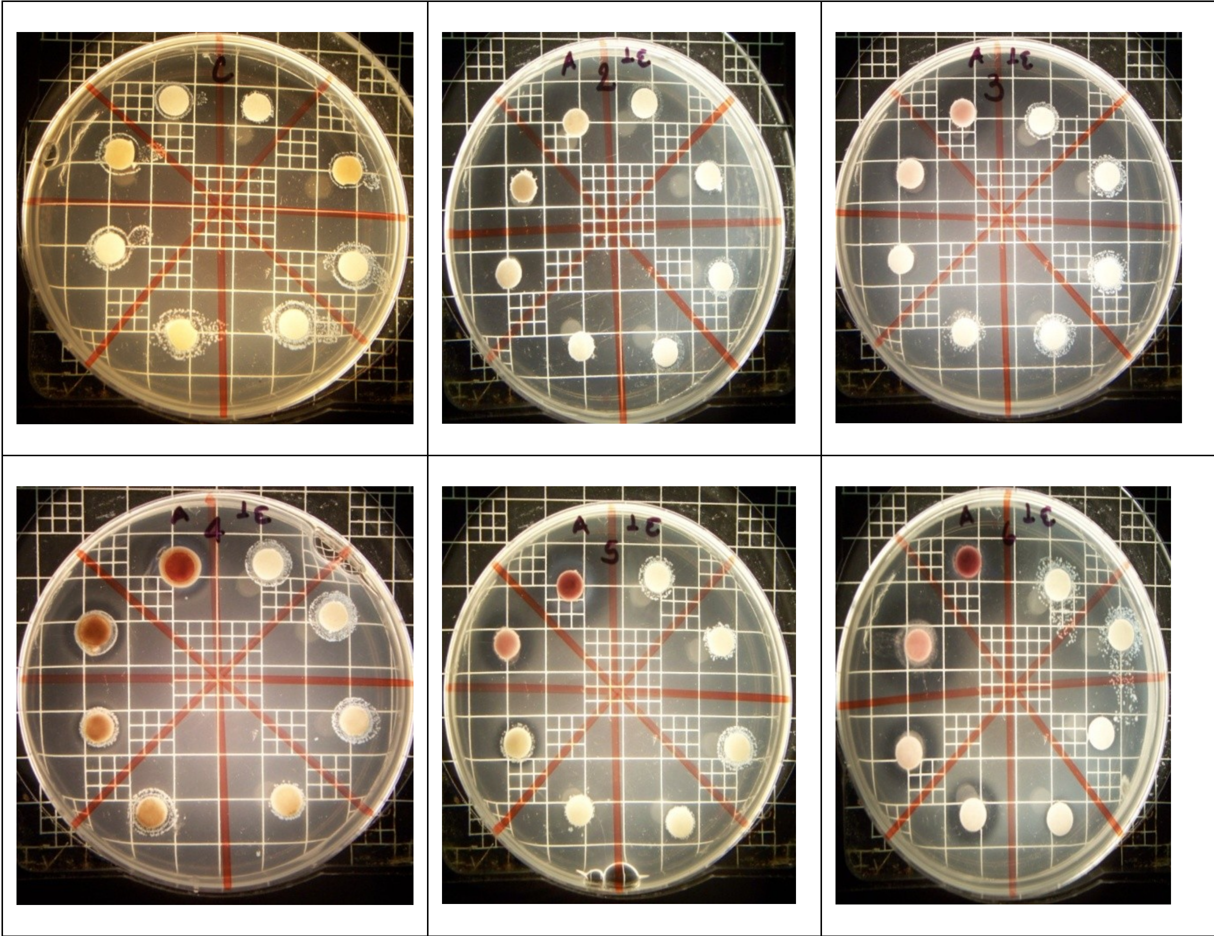
Amberlite Acetone *L. monocytogenes*



Ion exchange Methanol *L. monocytogenes*



Ion exchange Acetone *L.monocytogenes*



VITA

Lina M. Ramirez-Lopez

Candidate for the Degree of

Doctor of Philosophy

Thesis: CHARACTERIZATION OF PHENOLIC COMPOUNDS IN CYNTHIANA GRAPE (*Vitis aestivalis*)

Major Field: Animal Science/Food Science

Biographical:

Education:

Completed the requirements for the Master of Science in Food Science and Human nutrition at Clemson University, Clemson, SC, USA 2006. Thesis Title: Inactivation of thermo-resistant bacteria isolated from poultry offal.

Completed the requirements for the Bachelor of Science in Biochemistry at Caldas University, Manizales, Caldas, Colombia 2002. Thesis Title: Implementation of didactic tools for teaching biology and chemistry in high school. Honor thesis.

Experience:

Developed high performance liquid chromatography and mass spectroscopy methods for the identification and quantification of phenolic compounds

Built fractionation and purification systems for the isolation and purification of phenolic compounds using resin technology

Designed testing methodologies for measuring antibacterial properties of phenolic compounds in grape pomace

Isolated and identified *Lactobacillu*s from pork and beef

Determined antimicrobial properties of corn zein-coated polyethylene films

Studied the effect of lauric acid on corn zein films against *Listeria innocua* in hot dogs and bologna

Designed and conducted shelf life testing of fruits

Developed sensory analysis assay for retorted products

Applied culturing techniques to isolate and identify microorganisms

Developed experimental protocols for microbiological analyses

Calculated D, Z, and F values for thermally resistant bacteria from rendered materials

Evaluated hand washing techniques and sanitizer used for microbial decontamination

Identified spore forming bacteria in meat

Professional Memberships:

Institute of Food Technologists (2005-present)

American Chemical Society (2008-present)

Name: Lina M. Ramirez-Lopez Date of Degree: December, 2011

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: CHARACTERIZATION OF PHENOLIC COMPOUNDS IN CYNTHIANA GRAPE (*Vitis aestivalis*)

Pages in Study: 197 Candidate for the Degree of Doctor of Philosophy

Major Field: Food Science

Scope and Method of Study: Identification and quantification of the major phenolic compounds in the extracts of Cynthiana whole grape, juice, grape pomace and commercial dried grape pomace using High Performance Liquid Chromatography Comparative fractionation of phenolic compounds on Amberlite XAD 7 HP and cellulose ion exchange from grape pomace using acetone and methanol-water mixture as polar solvents by UV-Visible spectroscopy. Evaluation of antibacterial activities of phenolic compounds eluted on Amberlite XAD-7 HP and Ion exchange resins using acetone orr methanol in Cynthiana pomace.

Findings and Conclusions: Phenolic acids, flavanols, flavonols and stilbenes (PAFFS) were isolated from Cynthiana (*Vitis aestivalis*) whole grape, juice, or pomace. Flavonoid-anthocyanins (FA) were isolated using methanol/0.1% hydrochloric acid extraction. In addition, crude extractions of phenolic compounds from Cynthiana grape using 50% methanol, 70% methanol, 50% acetone, 0.01% pectinase, or petroleum ether were also evaluated. Reverse phase high performance liquid chromatography (RP-HPLC) with diode array detector was used to identify phenolic compounds. A method was developed for simultaneous separation, identification and quantification of both PAFFS and FA. For whole grape samples nine phenolic compounds were tentatively identified and quantified. The individual phenolic compounds content varied from 3 to 875 mg kg^{-1} dry weight. For juice, twelve phenolic compounds were identified and quantified. The content varied from 0.07 to 910 mg kg^{-1} dry weight. For pomace, a total of fifteen phenolic compounds were tentatively identified and quantified. The content varied from 2 mg kg^{-1} to 198 mg kg^{-1}. Results from HPLC analysis of the samples showed that gallic acid and (+ catechin hydrate were the major phenolic compounds in both whole grapes and pomace. Cyanidin and petunidin 3-*O*-glucoside were the major anthocyanins in juice. Extracts of methanol or acetone from Cynthiana pomace were applied to columns of Amberlite and Ion exchange resin and eluted with a mixture of $CH_3CN/TFA/H_2O$ (50:0.5:49.5 v/v/v). Phenolic compounds were obtained by collecting 5 mL fractions and subjected to ultraviolet (UV)/visible (vis) spectroscopy. The intense red fractions produced the highest relative concentration than the clear fractions at 280, 320, 370 and 520 nm. Antibacterial activities of the intense red fractions using spot on-the lawn- assay were studied on *E.coli, S.aureus* and *L.monocytogenes.* It was found XAD resin using acetone showed the highest inhibition against all the tested bacteria.

ADVISER'S APPROVAL: Christina A. Mireles DeWitt