IDENTIFICATIONS OF POLYPHENOLS AND QUANTIFICATION OF ANTHOCYANIDINS IN GRAPES AND GRAPE-DERIVED PRODUCTS

by

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And Approved by

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ABSTRACT OF THE THESIS

Identifications of Polyphenols and Quantification of Anthocyanidins

in Grapes and Grape-derived Products

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Polyphenols in grapes and grape-derived products have attracted public and scientific attention due to their numerous protective roles to human health. A rapid and comprehensive qualitative method was developed to characterize the different classes of polyphenols, such as anthocyanins, flavonols, phenolic acids and flavanols/proanthocyanidins, in grapes and grape-derived products. The detection was achieved by two runs with same HPLC gradient in different MS ionization modes and mobile phase modifiers (positive mode and 0.4% trifluoroacetic acid for anthocyanins and flavonols, negative mode and 0.1% formic acid for phenolic acids and flavanols). Under the optimized LC/MS conditions and based on the analysis of the MS and UV data and in comparison with the authenticated standards, a total of 53 polyphenolic compounds were successfully separated and individually identified including 33 anthocyanins, 12 flavonols, 4 phenolic acids and 4 flavanols/proanthocyanidins. With the

method developed, a survey was conducted to qualitatively assess and compare the composition of polyphenols among 29 grapes and grape-derived products. To facilitate the quantitation of the major class of polyphenolic anthocyanidins, a simple and precise acid assisted hydrolysis method was established for the quantitation of anthocyanidins in grape juice samples, grape berries and grape skins using LC/MS. Five most common anthocyanidins of delphenidin, petunidin, cyanidin, malvidin, and peonidin in the hydrolyzed grape extracts were included in the quantification study. The validation of this method showed that the recovery percentages of five anthocyanidins ranged from 98.59 % to 103.20% with the relative standard deviation (RSD) less than 5.03%. The qualitative method provided complete insight into the composition of polyphenols in grapes, and other grape-derived products. This quantitative method provides a rapid and accurate tool to quantitatively study individual anthocyanidin in grapes or grape juice samples for quality control and to facilitate the evaluation and comparison of new commercial grapes or grape juice products in market.

Keywords: Grape, LC-MS, Polyphenols, Anthocyanins, Flavonoids, proanthocyanidin, phenolic acid, Acid hydrolysis

DEDICATION

The thesis is dedicated to my husband, Dong, whose love, support and encouragement provided me the strength I needed to achieve my goals; and to our lovely daughter, Ella, whose happiness showed me the beauty of life.

It is dedicated to my parents and parents-in-law, for all of their support and guidance through my life.

It is also dedicated to my friends, for supporting me though this portion of my life and career.

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CHAPTER 1

INTRODUCTION

1.1 HIGH DEMAND OF PLANT-DERIVED DIETARY NUTRACEUTICALS CONTAINING POLYPHENOLS

An increasingly extraordinary high custom demand for plant-derived dietary nutraceuticals has arisen due to their beneficial role in managing health conditions, supporting a healthy immune system, reducing the risk of cardiovascular disease and cancer, and preventing memory related disorders, such as Alzheimer's disease. [1-4] The survey data indicate that 14 percent of Americans have taken an herbal product or nutraceutical in 2002 and that 18.9 percent have taken one or more dietary nutraceuticals in 2004. [5] Much of this custom demand for plant-derived dietary nutraceuticals is for products rich in polyphenols [4] which show healthy benefits such as anti-oxidation, anti-inflammation, antihistamine, antibacterial, anti-allergic, anti-platelet, antitumor and antiviral activities. [5] Data from Leatherhead Food International (LFI) shows that the world functional antioxidants market is increasing year on year by around 3 percent, and is valued at 400 million dollars in 2004 and 438 million dollars in 2007. Europe, U.S., and Japan account for 90 percent of this market. [6] Polyphenols from grapes and grape-derived products, such as beverages and dietary supplements, are also in high demand. Currently there are hundreds of grape polyphenol nutraceuticals on the market throughout the world. [7]

1.2 POLYPHENOLS IN GRAPES

Polyphenols, one group of the numerous and ubiquitous plant secondary metabolites, are integral part of both human and animal diets. They are widely distributed in variety of food grains such as sorghum, millet, barley, dry beans, peas, pigeon peas and other legumes, fruits such as apples, blackberries, cranberries, grapes, peaces, pears, plums, raspberries and strawberries, vegetables such as cabbage, celery, onion, as well as various medicinal plants and beverages. As secondary metabolites, polyphenols are not directly involved in the plant's growth and reproduction, but contribute to a myriad of important functions in plants such as protection from UV radiation, defense against invading pathogens, and attraction of pollinators and seed dispersers as well as the plant's characteristics such as taste, color or shelf life. [8-10]

By definition, natural polyphenols are chemical compounds characterized by the presence of more than one hydroxyl group on an aromatic ring. [11] According to their chemical structure, polyphenols can be divided into two kinds: flavonoids and nonflavonoids. [12, 13] The nonflavonoids include several major subgroups, such as hydroxycinnamic acids (e.g. caffeic acid) and derived lignans and cumarins, benzoic acids (e.g. gallic acid), hydrolyzable tannins (gallotannin), stilbenes (e.g. resveratrol). Another important and common group of polyphenols are flavonoids, which consist of flavanols, flavanones, flavones, isoflavones, flavonols, and anthocyanidins (listed in ascending order of oxidation), as well as quinones, a class of oxidized derivatives of polyphenols. [11] Although flavonoids' structure library is diversified, collectively considering many thousands of different flavonoids compounds, the characteristic structure of flanonoids is

the three-membered flavan ring system as shown in Figure 1. [14] The flavonoids in grapes and wines all have the same hydroxyl substitution groups in ring A wih 5, 7 dihydroxyl substitution. [13] Differences in the oxidation state and substitution groups on ring C define the different classes of the flavonoids. Flavans are defined by a saturated C ring. Flavones are defined by an unsaturated C ring between 2 and 3 position with a keto at the position 4. Anthocyanidins are defined by the fully aromatic ring with a positive charge of the oxygen in ring C. The –ol ending further specifies an alcohol substituent on the C ring, as in flavan-3-ol, in which the 3 indicates the position of hydroxyl group in the ring C. The major classes of flavonoids in grapes and grape-derived products are the flavanol/proanthochanidins (catechin), flavonols (quercetin), and anthocyanins (malvidin-3-glucose). [13]

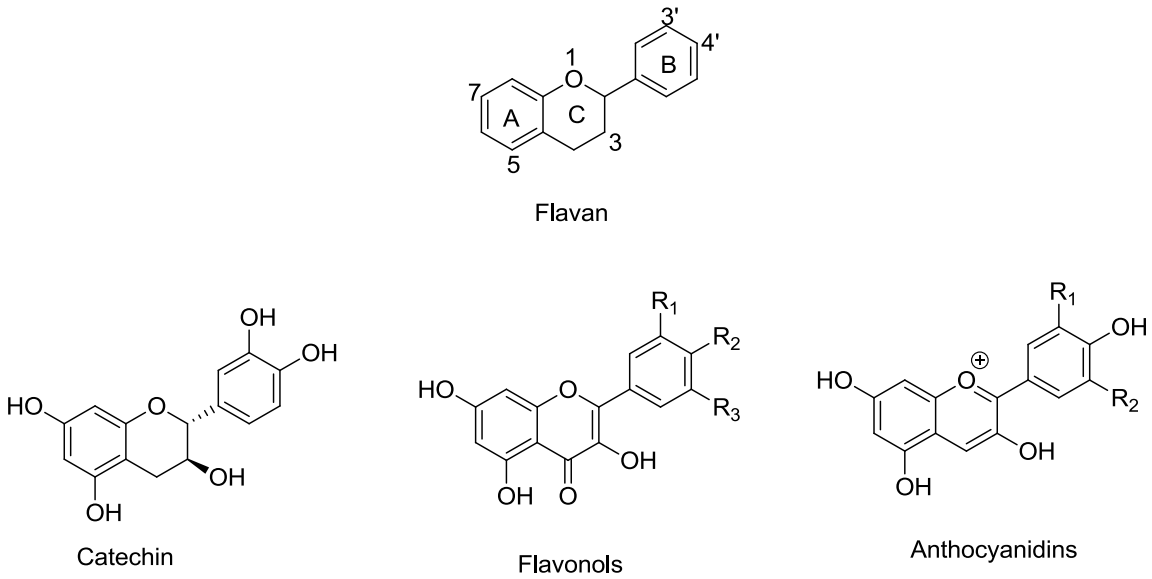
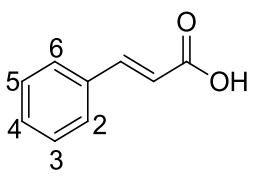


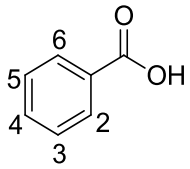
Figure 1. General structure of flavan and examples of different type of flavonoids

1.2.1 Phenolic acids

Phenolic acids are hydroxylated derivatives of cinnamic (C_6-C_3) and benzoic acids (C_6-C_1). The common hydroxycinnamic acid derivatives are *p*-coumaric acid, caffeic acid and ferulic acids (Figure. 2) which frequently occur in foods as simple ester with quinic acid or sugars. [15] Hydroxybenzoic acid has a general structure of C_6-C_1. Variations in the structures of individual hydroxybenzoic acids lie in the hydroxylation and methylation of the aromatic ring. Some common hydroxybenzoic acids include *p*-hydroxybenzoid acid, vanillic acid, gallic acid (Figure. 2). Gallic acid is a trihydroxyl derivative which participates in the formation of hydrolyzable gallotannins. Ellagic acid is dimeric condensation product of gallic acid. Ellagic acid usually participates in the formation of hydrolysable ellagitannins.



p-Coumaric acid 4 = OH
Caffeic acid 3 = 4 = OH
Ferulic acid 3 = OCH_3, 4 = OH



p-Hydroxybenzoid acid 4 = OH
Vanillic acid 3 = OCH_3, 4 = OH
Gallic acid 3 = 4 = 5 = OH

Figure 2. Chemical structures of some common phenolic acids

1.2.2 Stilbenes

Resveratrol is the principal stilbene in the grapes and is produced by several plants when under attach by pathogens such bacteria or fungi. Two forms of resveratrol exist including the *cis* and *trans* isomers (Figure. 3). The majority of the research papers and

scientific studies refer to resveratrol as trans-3, 5, 4'-trihydroxystilbene. Interestingly, light can cause the *cis*/*trans* isomerizations. [20]

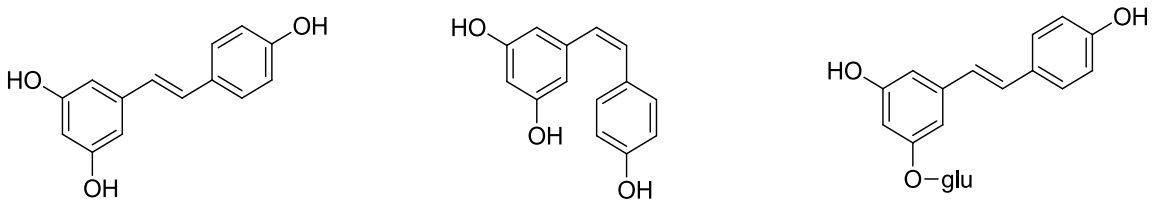


Figure 3. Chemical structures of *trans*-, *cis*-resveratrol and resveratrol glucoside

1.2.3 Flavanols

Flavanols are the most abundant class of flavonoids because the occurrences of stereoisomers in the 2 and 3 position of ring C as well as the formation of oligomers and polymers (proanthocyanidins or condensed tannins) with the condensation of flavan-3-ols. Two stereoisomers, (2R, 3S)-catechin and (2R, 3R)-epicatechin are illustrated in Figure 4 and both of the compounds have the 3', 4' catechol substitution on the B ring. Proanthocyanidin B1 is the dimmer with the condensation of catechin and epicatechin via 4→8 bond linkage (Figure. 4). In wines, proanthocyanidins contribute significantly to the complexity of the wine taste and mouth feel. [13]

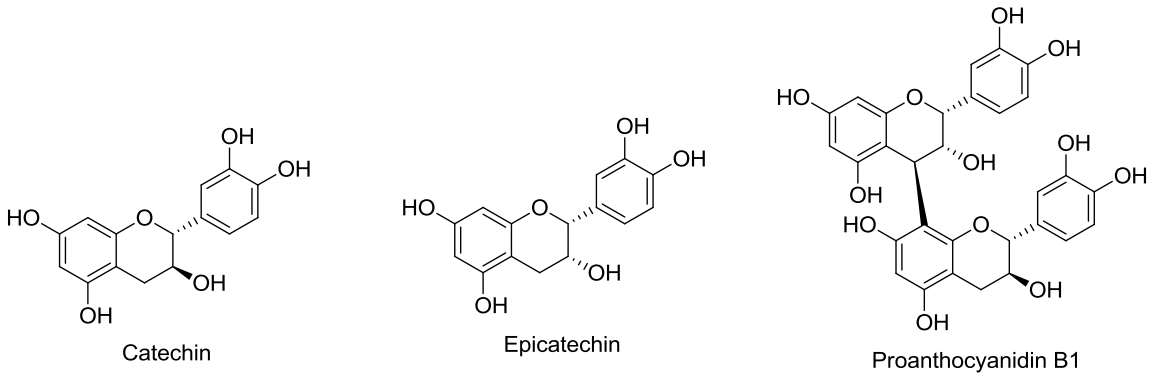


Figure 4. Chemical structures of *cis*-, *trans*- forms of flavan-3-ols and proanthocyanidin B1

1.2.4 Flavonols

Flavonols are one of the most extensively studied classes of polyphenols because of the abundand distribution and importance mainly relating to the antioxidant and other biological activities. [21] In grapes and wines, flavonols are mainly represented by simple aglycones (quercetin and myricetin) as well as some o-methylated derivatives such as isorhamnetin (quercetin 3'-methylether) as showing in Figure 5. The majority of flavonols are formed in conjugated with sugars, such as glucoside, galactoside and glucuronides. [22]

Biosynthetic pathways and flavonol compositions in the plant tissue are affected by sunlight, temperature and other conditions. For example, grape berries from sun exposed clusters were found to produce ten times more flavonol content compared with berries from shaded clusters. [23] The flavonol compositions are also associated with post harvest treatment, storage conditions and so on. For example, the exposure of Napoleon table grape in the ultraviolet radiation was shown to increase the flavonols content in the post

harvest treatment. When the Napoleon table grapes were stored at 0 ℃ for 10 days, the falvonol content was not affected. [24]

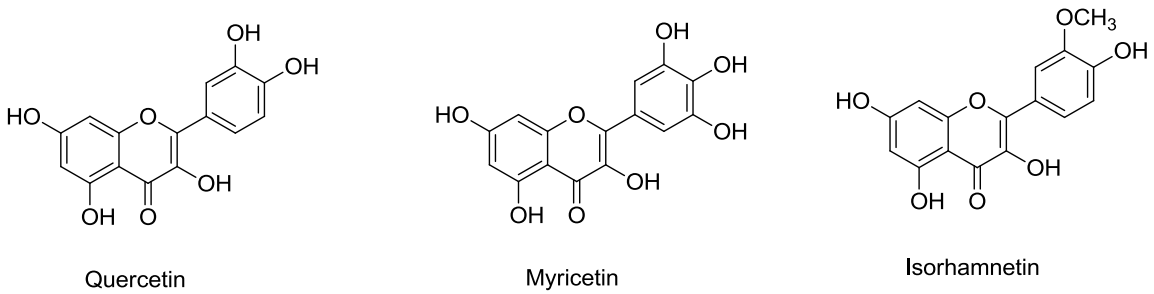


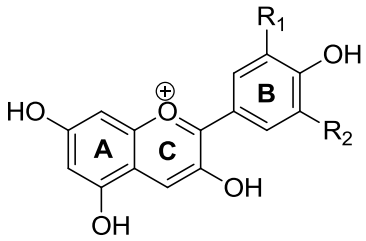
Figure 5. Structures of major flavonols in grapes and wines

1.2.5 Anthocyanins

Anthocyanins are versatile and red, blue or purple plant pigments that are widely distributed in plant tissues, mostly in flowers, fruits and vegetable, but also in leaves, stems and roots. [25]

Anthocyanidin is the aglycone of anthocyanin and it posseses the typical $C_6C_3C_6$ carbon skeleton of flavonoid. And they are distinguished from other flavonoids as a separate class by virtue of their ability to form flavylium cations (Figure. 6). Individual anthocyanidins are differentiated by the number of hydroxyl group and the degree of methylation on the flavylium salts. As a glycoside of anthocyanidin, anthocyanin is attached with one or more different sugars, such as glucose, galactose, rhamnose, arabinose, xylose and glucuronic acid at most often C-3, C-5, or C-7 positions. [11] In addition, diversity is further increased by the chemical combination of sugars with aromatic or aliphatic acid, such as acetic, succinic, caffeic acid and many more to

produce acylated anthocyanins. Considering all these factors, the variety and number of anthocyanins is large, leading to approximately 600 individual anthocyanins identified in the nature. [11, 26-28] The wide variety of anthocyanins coupled with their often chemical similarity results in a great number of peaks on chromatograms and difficulty in indentifying and separating individual anthocyanins. Given the enormous variety of anthocyanins, only a few reference compounds are commercially available, making it difficult to establish a definitive method to accurately quantify the anthocyanins composition and concentration in the grape and grape juice products. Scientists have been estimated the anthocyanins by choosing one reference glycoside for calibrations based on the fact that the chromatographic response of anthocyanins was very similar at 520 nm. [29] This method also has the limitation in that it can only be used when all the anthocyanins in the sample can be fully separated on a single base line. Otherwise the interference between inseparable peaks will either overestimate or underestimate the absolute quantities of compounds. Fortunately, the various anthocyanin glycoside patterns can be brought down to 23[27] naturally occurring anthocyanidins, among which the six most commonly anthocyanidins are delphinidin, cyanidin, pelargonidin, petunidin, peonidin and malvidin (Table 1).[11] Therefore, the problem can be overcomed by establishing a reliable quantification method that identifies and reveals the compositions of anthocynidins, the aglycone form of anthocyanins.



Anthocyanidin	Abbreviation	R_1	R_2
Cyanidin	Cy	OH	H
Delphinidin	Dp	OH	OH
Malvidin	Mv	OCH_3	OCH_3
Pelargonidin	Pg	H	H
Peonidin	Pn	OCH_3	H
Petunidin	Pt	OH	OCH_3

Figure 6. General structure of six most common anthocyanidins

1.3 PROTECTIVE ROLES OF GRAPE POLYPHENOLS IN VARIOUS DISEASES

In the recent decades, polyphenols from grapes, and grape-derived products have attracted a great deal of attentions due to the protective roles to the human health. To date, a number of scientists have conducted research on the protective roles of polyphenols extracted from grapes and grape derived products in various diseases. [30-34]

1.3.1 Grape polyphenols and neurodegenerative disease such as Alzheimer's disease

Alzheimer's disease (AD) is an incurable and devastating disease which is likely to become the single greatest threat to the heath of Americans. Alzheimer's Association reported that an estimated 5.3 million Americas of all ages have AD in 2010. One in ten people aged 65 and almost half of Americans over 85 are stricken with AD. [35] The number of Americans with AD and other dementias is increasing every year and will

continue to escalate rapidly in the coming years because of the longer life expectancies and aging of baby boomers. By 2050, it is estimated that 16 million Americans will be afflicted with AD, a more than 100 percent increase from 5.3 million Americans currently affected. [35]

Several hypotheses exist trying to explain the cause of the AD. Historically, cholinergic hypothesis proposed that AD was caused by reduced synthesis of neurotransmitter acetylcholine, [36] but the therapeutic strategies by blocking acetylcholine degradation produce only modest and temporary symptomatic effect. [30, 37-40] Two other popular hypotheses include β-amyloid (Aβ) hypothesis which postulates that AD pathology consists of deposition of Aβ peptides to form extracellular neuritic plaque (NP), and tau hypothesis which postulated that the AD is initiated by the aggregation of tau proteins to form neurofibrillary tangles (NFL). [30, 41] A more productive therapeutic strategy to treat or prevent AD was proposed to dissociate or prevent NP and/or NFL formation or deposition by preventing olygomerization of Aβ peptides and reducing tau species in the brain. [30] A commercial available grape seed polyphenolic extract enriched in proanthocyanidins (MegaNatural-AZ® GSPE), has illustrated its ability to attenuate the aberrant aggregation of Aβ by interfering with protofibril formation, preprotofibrilar oligomerization, and initial coil to α-helix/β-sheet secondary structure transitions. [30, 40] In the Tg2576 mouse model of AD, GSPE significantly attenuated AD-type cognitive deterioration and reduced cerebral amyloid deposition. [30, 42] GSPE also might benefit tau-mediated neuropathologic responses by inhibiting tau peptide aggregations, as well as dissociating preformed tau peptide aggregates in an *in vitro* model system. [30, 43, 44] Thus,

GSPE might benefit AD by simultaneously interfering with the two hallmark neuropathologies of the AD. [30] Red wine may also help reduce the relative risk of AD clinical dementia. [45-47] In a mouse model of AD, moderate consumption of red wine, Cabernet Sauvignon, significantly attenuated AD-type cognitive deterioration and Aβ neuropathology by reducing generation of AD-type Aβ peptides. [48] Additionally, treatment of a muscadine wine that characterized by distinct component composition of polyphenolic compounds, attenuated Aβ neuropathology and Aβ-related cognitive deterioration by interfering with the oligomerization of Aβ molecules to soluble high-molecular-weight Aβ oligomer species that are responsible for initiating a cascade of cellular events resulting in decline. [49] Therefore, there might be the possibility to develop a combination of dietary polyphenolic compounds for AD prevention and/or therapy by modulating muyltiple Aβ-related mechanism. [49] Based on the above evidence, efforts have focused on identifying the specific natural compounds in grapes, wine or other grape-related products that might be neuroprotective. [4] Resveratrol, a naturally occurring polyphenol found in grape skin and red wine, for example, has shown positive bioactivity mitigating potential AD by reducing Aβ, promoting intracellular Aβ degradation, [50, 51] and lowering Aβ accumulation by controlling AMP-activated protein kinase signaling. [52]

1.3.2 Grape polyphenols and cardiovascular disease

Cardiovascular disease is a leading cause of mortality among adults in Western countries. Several factors are considered to be the main cause for cardiovascular disease, such as cigarette smoking, high blood pressure, high serum total cholesterol and LDL-cholesterol, low serum HDL-choleserol, diabetes and advanced age. [53]

The cardiovascular benefits of red wine called great interest after the observation of "French Paradox", which was investigated by Fench epidemiologists [54, 55] in 1980s and strengthened by Renaud et al, who revealed that there was a low mortality rate from ischemic heart disease among French people despite their high consumption of saturated fats and the prevalence of other risk factors such as smoking. [56] This study drew great attention to the protective roles of wine against ischemic heart disease and further encouraged scientific research leading to the hypothesis that increased consumption of wine in France and other Mediterranean countries might be the explanation. [31]

Several review papers have demonstrated the promising roles of grape polyphenols against cardiovascular disease [31-33] in both animal and human model based on grape polyphenols' antioxidant properties, endothelia function, anti-platelet effects and so on. Antioxidant activities are mainly recognized as the ability to neutralize harmful free radical and to protect cells against the damaging effects of the reactive oxygen species such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxy nitrite. Polyphenols found in grapes have the capacity to scavenge reactive oxygen species. When fed to animals and humans, grape polyphenols have been shown to increase the

radical scavenging capacity of plasma. [57, 58] Grape polyphenols also were observed to have positive effects on leveraging the lipid profile parmeters (LDL-, HDL-cholesterol) by inhibiting oxidative modification of LDL. [32, 33] Besides antioxidant activity, grape polyphenols exhibited favorable endothelia function. In cultured endothelia cells, polyphenols from wine, grape juice, grape seed extract increased the activity of the endothelia isoform of nitric oxide synthase and stimulated the production of nitric oxide, a vasodilator that in the long term induced the protective genes for the cardiovascular system. [59, 60] Antiplatelet effects are another important function of grape polyphenols. In a human study, grape juice consumption for 14 days decreased platelet aggregation and superoxide production and increased nitric oxide production in healthy volunteers. [58]

1.3.3 Grape polyphenols and cancer

Cancer, one of the leading causes of death in the United States of America and many other countries over the world, poses both economic and psychological challenges to the society. [34, 61] Chemopreventive and anticancer activities of grape polyphenols have been investigated. [34, 62] For example, one of the grape-derived products, grape seed extracts (GSE), showed neoplastic efficacy in skin cancer, colorectal cancer, prostate cancer, breast cancer and more. [63] Proanthocyanidins, an important component in GSE, have shown promising activity against colon cancer Caco2 cells through the induction of apoptosis. [64] Scientists also found that proanthocyanidin dimmers, especially procyanidin B2 dimer inhibited the activity and expression of aromatase, an abnormal protein expressed in cancerous tissue.[65, 66] GSE was effective in preventing photocarcinogenesis at both initiation and promotion stages by mechanisms such as

antioxidant acitivity, inhibition of lipid peroxidation and more. [67, 68] The findings of these studies together suggest that grapes and grape-based products contain bioactive compounds that might potentially be developed as anticancer and cancer chemopreventive agents.

1.4 CONCLUSIONS AND OBJECTIVES OF THIS WORK

Grapes, grape juices, wines and other grape-derived products constituted an important source of dietary polyphenolic phytochemicals, including a large variety of both flavonoids and non-flavonoids constituents. [69] Over the past few years, a significant number of scientific papers and publications have focused on the analytical approaches on polyphenols in grapes and wine, and the numerous medical conditions that could be prevented or improved with the use of polyphenols. Though there has been a wide variety of research on grape polyphenols accumulated, several scientific gaps remain. Therefore the hypotheses and objectives of this work include:

1) Grape species vary and with each species, there is a wide number of diversity of cultivars. These genetic differences coupled with the recognition that the climate, post-harvest handling, storage and processing conditions will all impact the polyphenol profile and content in grapes and the grape related products. Therefore a precise assessment to compare the polyphenolic composition in various grapes and grape-derived products is critical. The aim of this work is to develop simple, effective and comprehensive LC/MS methods to characterize the composition and profile of polyphenolic compounds, such as phenolic acids, flavanols/proanthocyanins, flavonols and anthocyanins, in order to accurately

compare the polyphenol contents of different grape cultivals and grape-derived products and to evaluate parameters affecting the polyphenol compositions and accumulations during the production, harvest and storage.

2) We hypothesized that the polyphenol profiles in grapes and grape related products will vary. Therefore, based on the LC/MS method developed above, a survey was conducted to qualitatively compare the polyphenol profile in different grapes, various commercial grape juices and wines, and assessed the change of polyphenol compositions after the grapes are processed to wines.
3) Qualitative analysis is very important to identify and isolate each polyphenolic compounds in the samples, whereas a quantitative method is highly desirable to investigate the concentration of specific polyphenolic compounds in grapes and grape-derived products. Due to the large number and great diversity of anthocyanins in grapes, it is expensive and difficult to obtain all the standards and to quantify them individually. The estimation of anthocyanins by selecting one reference glycoside for calibration has its limitation because it can only be used when the all the anthocyanin peaks are fully separated. Fortunately, anthocyanins found in grapes and grape related products only derived six common aglycones of anthocyanidins. As such, a simple and accurate acid hydrolysis assisted method was developed to accurately quantitate the individual anthocyanidins compostion in grapes and grape juices and further more to compare the total content of anthocyanidins in various grapes berries/skins and commercial grape juices.

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CHAPTER 2

SURVEY OF POLYPEHNOL CONSTITUENTS IN THE GRAPES AND GRAPE DERIVED PRODUCTS

2.1 ABSTRACT

Polyphenols in grapes and grape-derived products have attracted public and scientific attentions due to their numerous protective roles to the human health. A rapid and comprehensive qualitative method has been developed to characterize the different classes of polyphenols, such as anthocyanins, flavonols, phenolic acids and flavanols/proanthocyanidins, in grapes and grape-derived products. The detection was achieved by two runs with same LC gradient in different MS ionization modes and mobile phase modifiers (positive ionization mode and 0.4% trifluoroacetic acid for anthocyanins and flavonols; negative ionization mode and 0.1% formic acid for phenolic acids and flavanols). Based on analyzing the MS and UV data and in comparison with the authenticated standards, a total of 53 compounds were identified, including 33 anthocyanins, 12 flavonols, 4 phenolic acids and 4 flavanols/proanthocyanidins. With the method developed, a survey was conducted to qualitatively assess and compare the composition of polyphenols among 29 grapes and grape derived samples. The qualitative method provided complete insight into the compositions of polyphenols in grapes, and other grape-derived products.

2.2 INTRODUCTION

Grapes, and grape-derived products are rich in various bioactive dietary polyphenols [1-3] Polyphenols can be divided into two kinds: flavonoids and nonflavonoids. [4, 5] The nonflavonoids include several major subgroups, such as hydroxycinnamic acids (e.g. caffeic acid) and derived lignans and cumarins, benzoic acid (e.g. gallic acid), hydrolyzable tannins (gallotannin), stilbenes (e.g. resveratrol). Another important and common group of polyphenols found in grapes and grape derived products include the flavonoids, which consist of flavanols, flavanones, flavones, isoflavones, flavonols, and anthocyanidins (listed in ascending order of oxidation), as well as quinones, a class of oxidized derivatives of polyphenols. [2] Although flavonoids' structure library is extensively diversified, collectively encompassing thousands of different flavonoids compounds, the characteristic structure of flanonoids is the three-membered flavan ring system as shown in Figure 7. [6] The flavonoids in grapes, grape juice products and wines all have the same hydroxyl substitution groups in ring A. [5] Differences in the oxidation state and substitution groups on ring C define the different classes of the flavonoids. Flavans are defined by a saturated C ring. Flavones are defined by an unsaturated C ring between 2 and 3 position with a keto at the position 4. Anthocyanidins are defined by the fully aromatic ring with a positive charge of the oxygen in ring C. The –ol ending further specifies an alcohol substituent on the C ring, as in flavan-3-ol, in which the 3 indicates the position of hydroxyl group in the ring C. The major classes of flavonoids in grapes and grape-derived products are the flavanol/proanthochanidins (catechin), flavonols (quercetin), and anthocyanins (malvidin-3-glucose). [5]

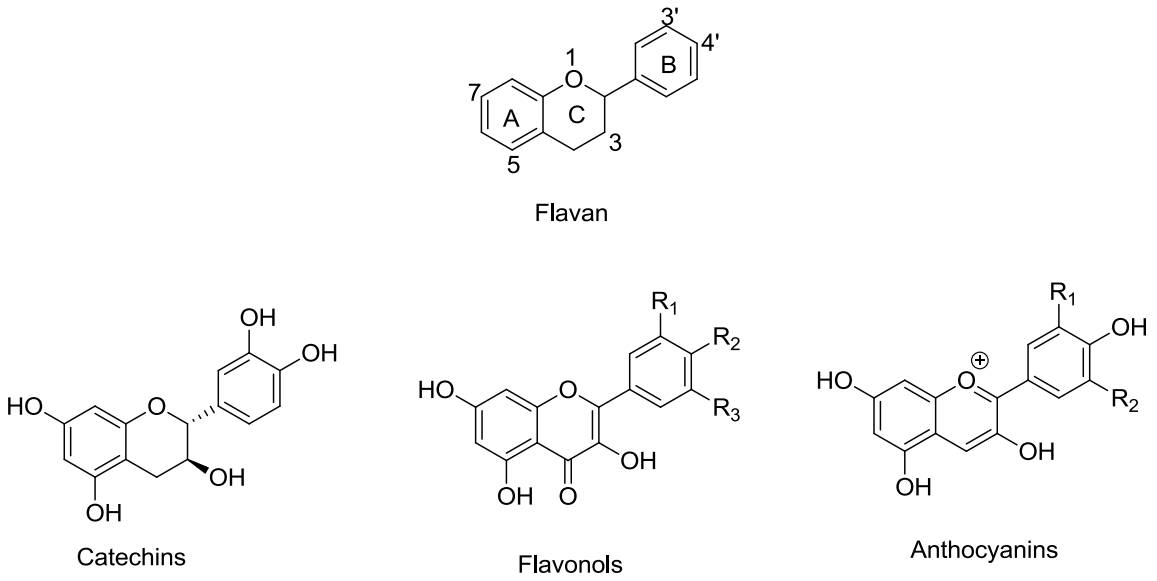

Figure 7. General structure of flavan and examples of flavonoids

Polyphenols from grapes and grape-derived products have attracted a great deal of attention due to their numerous protective roles to various diseases, such as cardiovascular diseases, neurodegenerative diseases and cancers. [7-11] For example, the prevention and amelioration of one of the neurodegenerative diseases, Alzheimer's disease (AD), was shown to be associated with the administration of grapes and grape derived supplements in the epidemiological studies. [12-15] Several hypotheses exist trying to explain the cause of the AD. Historically, cholinergic hypothesis proposed that AD was caused by reduced synthesis of neurotransmitter acetylcholine, [16] but the therapeutic strategy by blocking acetylcholine degradation are not preventive or curative and produce only a modest and temporary symptomatic effect. [7, 17-20] Two other popular hypotheses include β-amyloid (Aβ) hypothesis which postulates that AD pathology is deposition of Aβ peptides to form extracellular neuritic plaque (NP), and tau hypothesis which postulated that the AD is initiated by the aggregation of tau proteins to form

neurofibrillary tangles (NFL). [7] Later, another therapeutic strategy to treat or prevent AD was proposed to dissociate or prevent NP and/or NFL formation or deposition by preventing olygomerization of Aβ peptides and reducing tau species in the brain. [7, 21] A commercial available grape seed polyphenolic extract (GSPE), MegaNatural-AZ (MN), was found to attenuate the aberrant aggregation of Aβ by interfering with protofibril formation, preprotofibrilar oligomerization, and initial coil to α-helix/β-sheet secondary structure transitions. [7, 20] In the Tg2576 mouse model of AD, GSPE significantly attenuated AD-type cognitive deterioration and reduced cerebral amyloid deposition. [7, 22] GSPE also might benefit tau-mediated neuropathologic responses by inhibiting tau peptide aggregations, as well as dissociating preformed tau peptide aggregates in an *in vitro* model system. [7, 23-24] Thus, GSPE might benefit AD by simultaneously interfering with the two hallmark neuropathologies of the AD. [7] Red wine may also help reduce the relative risk of AD clinical dementia. [25-27] In a mouse model of AD, moderate consumption of red wine, Cabernet Sauvignon, significantly attenuated AD-type cognitive deterioration and Aβ neuropathology by reducing generation of AD-type Aβ peptides. [28] Additionally, treatment of a muscadine wine that characterized by distinct component composition of polyphenolic compounds, attenuated Aβ neuropathology and Aβ-related cognitive deterioration by interfering with the oligomerization of Aβ molecules to soluble high-molecular-weight Aβ oligomer species that are responsible for initiating a cascade of cellular events resulting in cognitive decline. [29] Therefore, there might be the possibility to develop a combination of dietary polyphenolic compounds for AD prevention and/or therapy by modulating muyltiple Aβ-related mechanism. [29] Based on this evidence, efforts have focused on identifying compounds in grapes or other grape-

related products that might be neuroprotective. [30] Resveratrol, a naturally occurring polyphenol found in grape skins and red wine, also has exhibited therapeutic effects for the AD by reducing Aβ, promoting intracellular Aβ degradation, [31, 32] and lowering Aβ accumulation by controlling AMP-activated protein kinase signaling. [33]

In the recent decade, significant research has focused on polyphenol compositions in grapes, grape seed extracts and wines. [34-41] Most of the efforts focused on either one type of grape products or one specific category of polyphenols or anthother of natural prodcuts. Grape species vary and within each species, there is a wide number and diversity of cultivars. These genetic differences coupled with the recognition that the climate, post-harvest handling, processing and storage conditions all impact the polyphenol profile and content in grapes and grape-derived products. Therefore a precise assessment to determine the polyphenolic composition in various grapes and grape-derived products is critical. The aim of this work is to develop simple, effective and comprehensive LC/MS methods to characterize the profile of polyphenolic compounds, such as phenolic acids, flavanols/proanthocyanidins, flavonols and anthocyanins. Based on the LC/MS method developed above, a survey would then be conducted to qualitatively compare the polyphenol profiles in different grapes and various commercial grape juices as well as assess the change of polyphenol compositions after the grapes are processed to wines.

2.3 MATERIALS AND METHODS

2.3.1 Materials

Standard compounds caffeic acid, 4-hydroxybenzoic acid, (-)-Epicatechin, p-coumaric acid, and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Procyanidin B2, caftaric acid and cyanidin-3-glucoside were purchased from ChromaDex (Irvine, CA). The HPLC grade acetonitrile (ACN), methanol (MeOH), trifluoroacetic acid (TFA) were obtained from Fisher Scientific Co. (Fair Lawn, NJ). HPLC grade formic acid was obtained from Acros Organics (NJ). HPLC-grade water was prepared using a Millipore Milli-Q purification system (Millipore Corporation, Bedford, MA). Fifteen retail grape juices were purchased from several food super markets and they covered brands, such as Welch's®, Langers®, Healthy Balance®, Wild harvest®, Shopper Value®, ACME®, ShopRite®, Santa Cruz®, Snapple®, Walgreens®, Manischewitz® and Kedem® as shown in Table 1. Welch's Concord Grape Juice 100% concentrated (originally from Welch's Inc.), Cabernet Franc Grape berries and Noriet Grape berries as a whole fruit were provided by Purdue University. Pandal Red Seedless Table Grape, Pandal Black Seedless Table Grape and other three grape-produced dietary supplements (sample # 27, 28, 29 in Table 1) were commericially purchased. The grape skins were manually peeled from fresh grape berries. All grape juices and wines were stored at 4 ℃ and grapes berries and skins were stored in - 20 ℃.

Table 1. Information of experimental samples of grapes and grape-derived products

Sample code	Sample name	Internal QC Ref. Num.	Original source
1	Welch's Concord Grape Juice 100% concentrated	BC/0902200023	Purdue Univ.
2	Welch's Concord Grape Juice Cocktail	NAU1010010002	ACME
3	Welch's Light Concord Grape Juice Beverage	NAU1010010003	ACME
4	Welch's 100% Juice Black Cherry Concord Grape Juice	NAU1010010004	ACME
5	Langers Pomegranate Grape Juice	NAU1010010006	ACME
6	Healthy Balance Grape Juice	NAU1010010007	ACME
7	Wild Harvest Organic Grape Juice	NAU1010010008	ACME
8	Shoppers Value Grape Drink	NAU1010010009	ACME
9	ACME Grape Juice Cocktail	NAU1010010010	ACME
10	ShopRite Pasteurized Grape Juice	NAU1010010011	ShopRite
11	Santa Cruz Organic Concord Grape Juice	NAU1010010012	ShopRite
12	Snapple Naturally Flavored Grapeade Juice	NAU1010010013	ShopRite
13	Walgreens Grape Juice	NAU1010040014	Walgreens
14	Manischewitz Premium Grape Juice	NAU1010060015	A&P
15	Kedem Concord Grape Juice	NAU1010060016	A&P
16	Cabernet Franc Grape Berries	BC/0810140001	Purdue Univ.
17	Cabernet Franc Grape Skins	BC/0810140002	Purdue Univ.
18	Cabernet Franc Wine	BC/0811180004	Purdue Univ.
19	Noiret Grape Berries	BC/0810140005	Purdue Univ.
20	Noiret Grape Skins	BC/0810140006	Purdue Univ.
21	Noriet Wine	BC/0811180008	Purdue Univ.
22	Cabernet Sauvignon Wine	BC/1118080020	Purdue Univ.
23	Pandol Red Seedless Table Grape Berries	NAU1010090017	Costco
24	Pandol Red Seedless Table Grape Skins	NAU1010090018	Costco
25	Pandol Black Seedless Table Grape Berries	NAU1010090019	Costco
26	Pandol Black Seedless Table Grape Skins	NAU1010090020	Costco
27	Grape Complete With Pine Bark	NAU1010090021	Country Life
28	Best French Red Wine	NAU1010090022	Doctor's Best
29	Herbal Actives Red Wine	NAU1010090023	Nature's Plus

2.3.2 Sample preparations

For qualitative identification, the grape wines and grape juices (sample # 1-15, 18, 21 and 22 in Table 1) were filtered through 0.45 μm filter into HPLC vials and directly injected for LC/MS analysis. The grape berries (sample # 16, 19, 23 and 25 in Table 1) as a whole fruit were frozen in – 20 ℃ and then grounded into small pieces. Around one gram of grape berries were extracted with 10 mL 70% MeOH containing 1% acetic acid solution and sonicated for 20 minutes. The extraction was conditioned to room temperature, and then approximately 1 mL samples were filtered through 0.45 μm filter and transferred into HPLC vials. Grape skins (sample # 17, 20, 24 and 26 in Table 1) were manually peeled from fresh grape berries and grouded and then prepared using the same procedure as for grape berries. Around 100 mg of grape-produced dietary supplements (sample # 27-29 in Table 1) were dissolved in 70% MeOH containing 1% acetic acid solution and sonicated for 20 min. The extraction was conditioned to room temperature and then aound 1 mL was filtered into vials through 0.45um filter into HPLC vials prior to the injection.

2.3.3 Equipment and HPLC-MS condition

HPLC separation was performed on a Polaris amide-C18 column, 250 x 4.6mm, 5 μM (Varian Inc.). For LC-MS analysis, a Hewlett Packard Agilent 1100 Series LC/MS (Agilent Technologies, Waldbronn, Germany) equipped with autosampler, quaternary pump system, DAD detector, degrasser, MSD trap with an electrospray ion source (ESI), and software of HP ChemStation, Bruker Daltonics 4.2 and Data Analysis 4.2 was used. The indentification of anthocyanins, flavonols, phenolic acids and flavanols were

achieved using the same LC gradient, but with different MS ionization mode and mobile modifiers. Anthocyanins and flavonols were detected under positive ion mode with 0.4% TFA (v/v) in water and ACN. Phenolic acids and proanthocyanidins were detected under negative ion mode with 0.1% FA (v/v) in water and ACN. HPLC separation was performed with the mobile phase containing solvent A (0.4% TFA or 0.1% FA in water) and B (0.4% TFA or 0.1% FA in ACN) in gradient: 0-20 min, linear gradient from 10 % to 20 % B; 20-30 min, linear gradient from 20% to 30% B; 30-40 min, isocratic elution at 30% B; 40-50 min, linear gradient from 30% to 50%; 50-60 min, linear gradient from 50% to 60%. The flow rate was set at 1.0 mL/min. The injection volume was 20 μL and the UV detector was set at 254, 280, 370, 520 nm. The eluent was monitored by electrospray ion mass spectrometer (ESI-MS) under positive ion mode for anthocyanins and flavonols, and under negative ion mode for phenolic acids and proanthocyanins. The samples were scanned from *m/z* 100 to 900. ESI was conducted by using a needle voltage of 3.5KV (positive) and -3.5KV (negative). High-purity nitrogen (99.999%) was used as dry gas and at a flow rate of 12 L/min capillary temperature at 350 ^{o}C. Nitrogen was used as nebulizer at 60 psi and Helium as collision gas.

2.4 RESULTS AND DISCUSSIONS

With the method developed, a survey on the polyphenol compositions of 29 samples, including, grape berries, grape skins, grape juices, grape wines and grape-produced dietary supplements was conducted. Same LC gradient but different MS ion mode and mobile phase modifier were applied to detect different subgroup of polyphenols (positive ion mode and 0.4% TFA for anthocyanins and flavonols; negative ion mode and 0.1% FA

for phenolic acids and flavanols). Under the optimized LC/MS conditions and based on analyzing the MS and UV data and incomparison with the authenticated standards, a total of 53 compounds, including 33 anthocyanins, 12 flavonols, 4 phenolic acids and 4 flavanols/proanthocyanidins were successfully separated and indentified.

2.4.1 Anthocyanin identification and profile comparison

The representative UV chromatograms at 520 nm of Welch's Concord Grape Juice 100% concentrated (sample # 1 in Table 1) and Cabernet Franc Wine (sample # 18 in Table 1) are illustrated in Figure 8. The identities, retention time, peak assignment, molecular ions and the characteristic fragment ions for individual compound are listed in Table 2. Based on the analysis of MS and UV data and in comparison with authenticated standards, a total of 33 anthocyanins were simultaneously identified as anthocyanidin diglycosides, glucoside, acetylglucoside, coumaroylglucoside, coumaroyldiglucoside and anthocyanin pyruvate derivatives (Table 2). [34-36] The structures and fragment pathway of selected anthocyanins (Cmpd. 24 and 32) are illustrated in Figure 10. The representative MS spectra of cyanidin derivatives (Cmpd. 2, 8, 15, 17, 24) detected in Welch's Concord Grape Juice 100% concentrated and malvidin derivatives (Cmpd. 30, 31, 32, 33) detected in Cabernet Franc Wine are showed in Figure 9.

Table 2. Peak assignments for the analysis of grapes and grape derived products

Peak	t_R (min)	Identities	Molecular and fragment ions (*m/z*)	Cmpd. code
1	11.7	Dp-G-G	627, 465, 303	1
2	13.6	Cy-G-G	611, 449, 287	2
3	14.3	Pt-G-G	641, 479, 317	3
4	15.4	Pg-G-G	595, 433, 271	4
5a	16.1	Pn-G-G	625, 463, 301	5
5b	16.5	Mv-G-G	655, 493, 331	6
6	17.1	Dp-G	465, 303	7
7	19.6	Cy-G	449, 287	8
8	20.1	Pt-G	479, 317	9
9	21.8	Pg-G	433, 271	10
10	22.6	Pn-G	463, 301	11
11	22.9	Mv-G	493, 331	12
12	26.4	Dp-G-Ac	507, 303	13
13a	28.2	Dp-G-G-Co	773, 611, 465, 303	14
13b	28.3	Cy-G-Ac	491, 287	15
13c	28.5	Pt-G-Ac	521, 317	16
14a	29.6	Cy-G-G-Co	757, 595, 449, 287	17
14b	29.7	Pt-G-G-Co	787, 625, 479, 317	18
15a	30.1	Pn-G-Ac	505, 301	19
15b	30.1	Mv-G-Ac	535, 331	20
16a	30.7	Mv-G-G-Co	801, 639, 493, 331	21
16b	31.0	Pn-G-G-Co	771, 609, 463, 301	22
17	32.9	Dp-G-Co	611, 303	23
18	34.4	Cy-G-Co	595, 287	24
19	34.5	Pt-G-Co	625, 317	25
20	35.9	Mv-G-Co	639, 331	26
21	36.5	Pn-G-Co	609, 301	27
22	16.8	Dp-G-Py	533, 371	28
23	20.1	Pt-G-Py	547, 385	29
24	23.2	Mv-G-Py	561, 399	30
25	26.2	Mv-G-Ac-Py	603, 399	31
26	32.0	Mv-G-Co-Py	707, 399	32
27	36.0	Mv-G-VP	609, 447	33

28	25.2	Myricetin-G	481, 319	34
29	26.0	Myricetin-GR	495, 319	35
30	28.8	Quercetin-G	465, 303	36
31	29.4	Quercetin-GR	479, 303	37
32	29.8	Syringetin-G	509, 347	38
33	31.8	Isorhamnetin-GR	493, 317	39
34	34.0	Syringetin-G-Ac	551, 347	40
35	41.0	Myricetin	319	41
36	48.5	Laricitrin	333	42
37	51.6	Quercetin	303	43
38	52.1	Syringetin	347	44
39	54.7	Isorhamnetin	317	45
40	5.7	Gallic acid	169	46
41	11.7	PAC dimmer	577	47
42	13.1	Catechin	289	48
43	15.2	Vanillic acid	167	49
44	16.3	Epicatechin	289	50
45	16.7	PAC dimmer	577	51
46	18.5	Caffeic acid	179	52
47	26.8	*p*-Coumaric acid	163	53

Note: The phenolic acids (gallic acid, vanillic acid, caffeic acid, p-coumaric acid), proanthocyanidins, and cyanidin-3-glucoside were compared with the retention time of the authenticated standard. Dp: delphenidin, Pt: petunidin, Cy: cyanidin, Mv: malvidin, Pn: peonidin, Pg: pelargonidin, G: Glucosyl or galactosyl moiety, GR: Glucuronosyl, Ac: acetyl, Co: coumaroyl, Py: pyruvate, VP: vinylphenol, PAC: proanthocyanidin. For the flavonoid glycosides, in general glucosyl group, occasionally galactosyl group, was substituted on the 3/5 position of aglycone and acetyl/coumaroyl group was linked to 6’ position of sugar moiety).

Table 3. The presence of anthocyanins in individual grape sample

Cmpd. Code	Sample Code																												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1	+	+	+	+	+	+	+	+	T	+	+	+	+	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-
2	+	+	+	+	+	+	+	+	T	+	+	+	+	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-
3	+	+	+	+	+	+	+	+	T	+	+	+	+	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-
4	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	-	-	-	-	-	-	-	-	-
5	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-
6	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-
7	+	+	+	+	+	+	+	+	+	+	+	T	+	T	+	+	+	+	+	+	+	T	T	T	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	T	+	T	+	+	+	-	+	+	T	-	T	T	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	T	+	-	+	+	+	+	+	+	+	T	+	+	+	+	+	+	+
10	T	T	T	T	T	-	T	T	-	T	T	T	-	-	T	T	T	-	T	T	-	-	T	T	T	T	T	T	T
11	+	+	+	+	+	+	+	+	T	+	+	+	+	-	+	+	+	T	+	+	T	-	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	T	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	+	-	+	+	+	+	+	-	-	+	+	-	+	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-
14	+	+	+	+	+	+	+	-	-	+	+	T	T	-	+	-	-	-	T	T	T	-	-	-	-	-	-	-	-
15	+	T	+	+	+	+	+	-	-	+	+	-	-	-	+	+	+	T	+	+	+	-	-	-	-	-	-	-	-
16	+	T	+	+	+	+	+	-	-	+	+	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
17	+	+	+	+	+	+	+	+	T	+	+	T	+	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-
18	+	+	+	+	+	+	+	+	T	+	+	T	+	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-
19	+	-	+	T	T	+	+	-	-	T	+	-	-	T	+	+	+	+	+	+	+	-	T	T	+	+	-	-	-
20	+	-	+	T	T	+	+	-	-	T	+	-	T	-	+	+	+	+	+	+	+	-	-	-	+	+	-	-	-

21	+	+	+	+	+	+	+	+	T	+	+	+	+	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-
22	+	+	+	+	+	+	+	+	T	+	+	+	+	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-
23	+	+	+	+	+	+	+	T	T	+	+	-	T	T	+	+	+	-	+	+	+	-	-	-	+	+	T	T	T
24	+	+	+	+	+	+	+	-	-	+	+	-	+	-	+	+	+	-	+	+	+	-	+	+	+	+	-	-	-
25	+	+	+	+	+	+	+	-	-	+	+	-	+	-	+	+	+	-	+	+	+	-	T	T	+	+	T	T	T
26	+	+	+	+	+	+	+	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+	T	T	+	+	+	+	+
27	+	-	+	+	+	+	+	-	-	+	+	-	T	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	T	T	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	+	+	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	T	+	-	-	-	-	T	T	+
31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	T
33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	T	+	-	-	-	-	-	-	-
Total	**26**	**22**	**26**	**26**	**27**	**25**	**26**	**16**	**15**	**26**	**26**	**16**	**22**	**4**	**26**	**16**	**16**	**16**	**27**	**27**	**29**	**10**	**11**	**11**	**13**	**13**	**11**	**11**	**12**

Sample codes are same as in Table 1. Compound codes refer to Table 2. (+) Present, (-) not detectable, T: trace, Total: total number of anthocyanins present in individual samples.

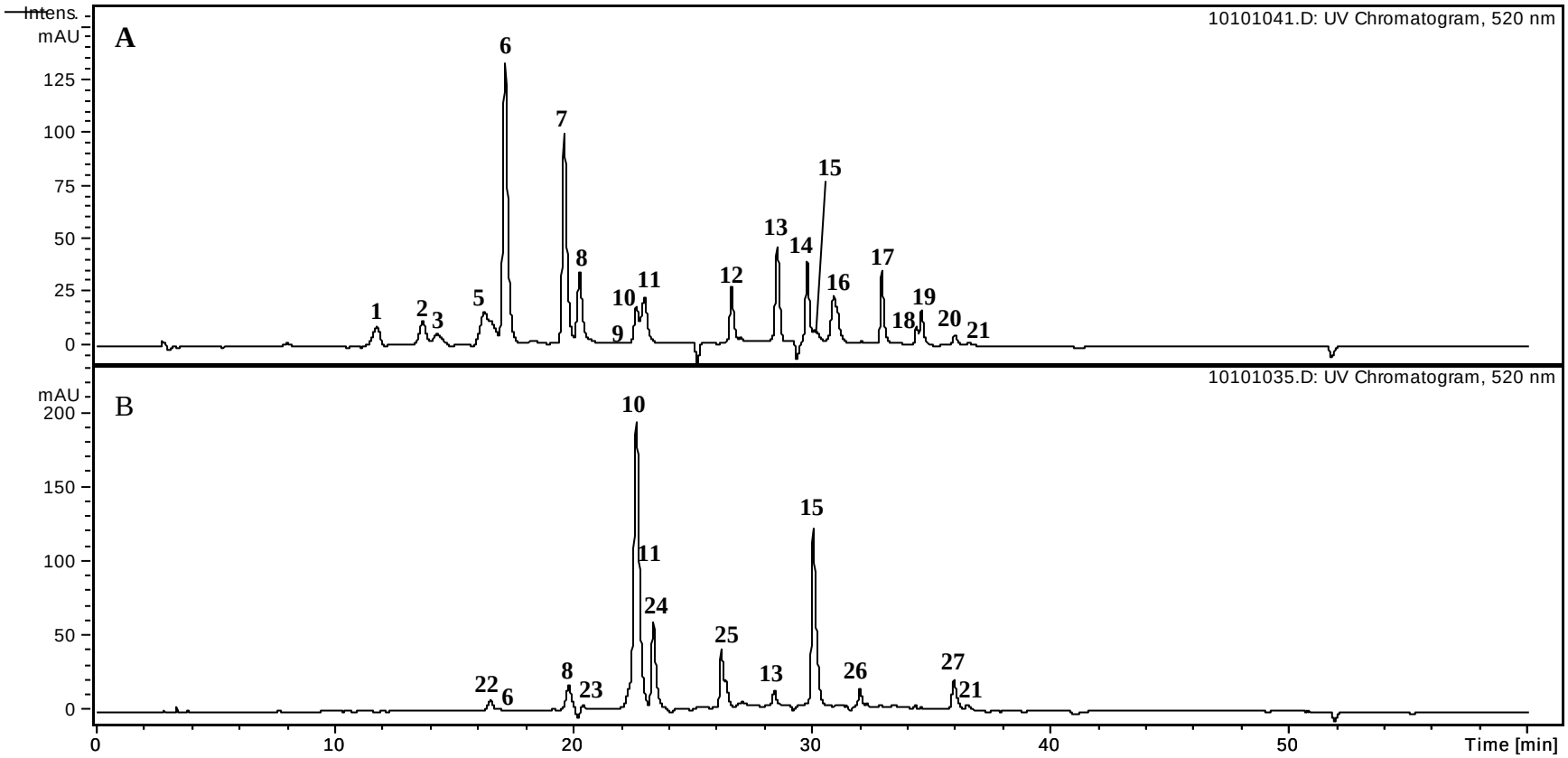

Figure 8. Representative UV chromatograms (520 nm) of (A) Welch's Concord Grape Juice 100% concentrated and (B) Cabernet Franc Wine. Peak assignment, t_R, and identities are listed in Table 2. The presence of anthocyanin profile for each sample is listed in Table 3.

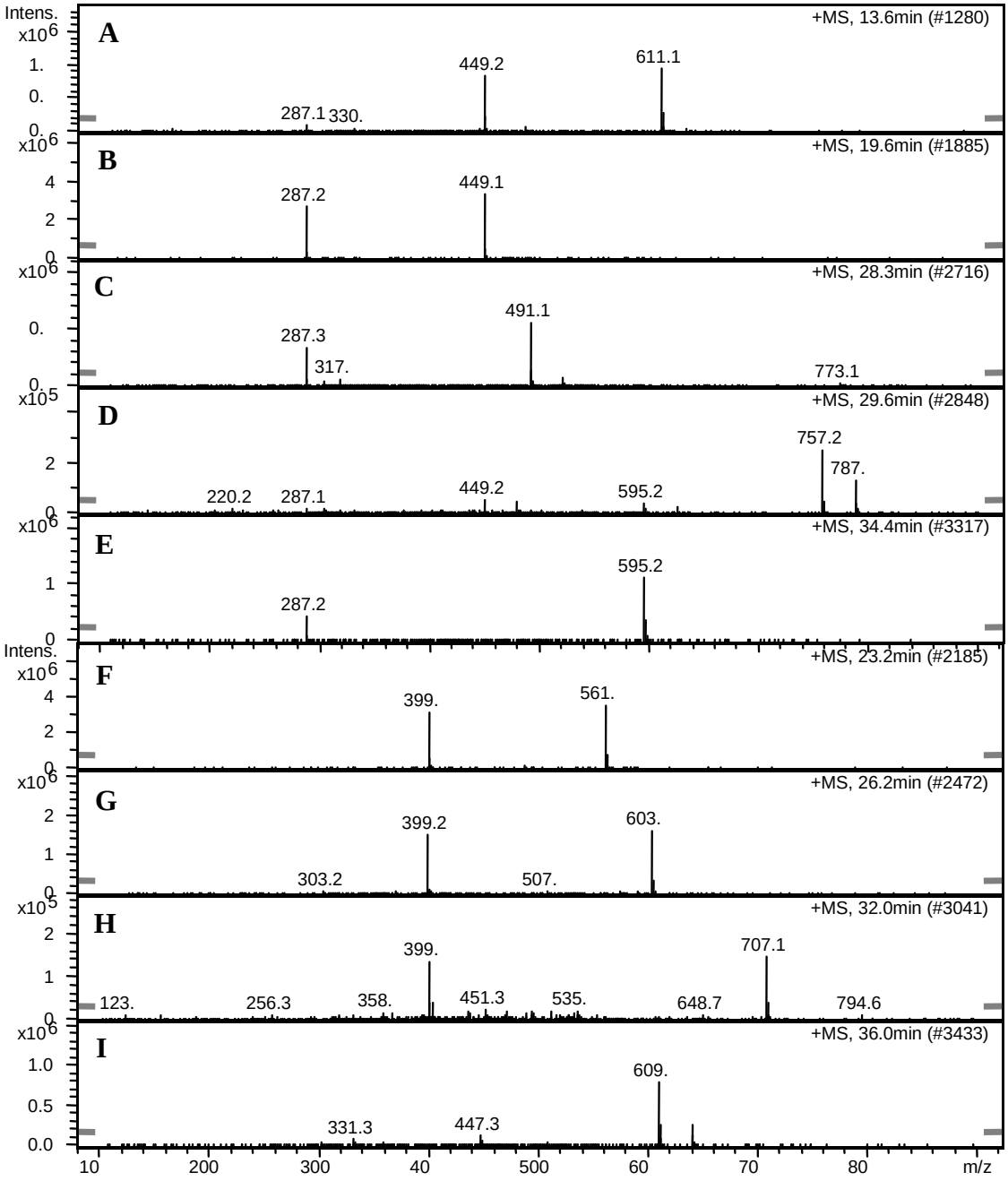

Figure 9. Representative MS spectra of cyanidin derivatives of Cmpd. 2 (A), 8 (B), 15 (C), 17 (D) and 24 (E) in Welch's Concord Grape Juice 100% concentrated and malvidin pyruvate derivatives of Cmpd. 30 (F), 31 (G), 32 (H) and 33 (I) in Cabernet Franc Wine.

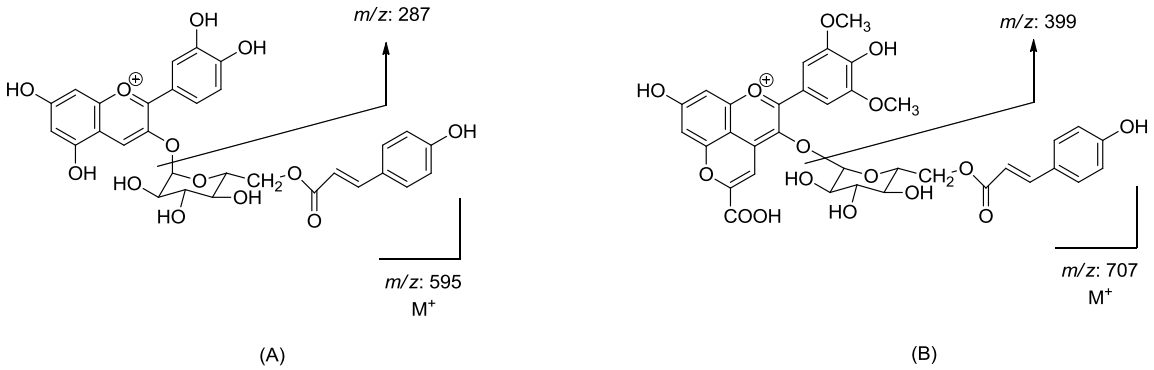

Figure 10. Structures of selected anthocyanins of (A) Comp. 24 (MS spectrum is shown in Figure 9E) and (B) Comp. 32 (MS spectrum is shown in Figure 9H) and their fragment pathway.

Previous research has identified many anthocyanins in grapes and grape-derived products. [34-36] In general, the glucosyl group, and occasionally galactosyl group, is substituted on the 3 and 5 positions of aglycone. The acetyl and coumaroyl groups are attached on the 6' position of the sugar moiety. Based on the UV spectrum and molecule ions and their corresponding fragment ions, most of the structures could be determined. For example, MS spectra (Figure. 9A) of Cmpd. 2 (t_R, 13.6 min) indicates that it has molecular ion at *m/z* 611 and is fragmented to *m/z* 499 ([M-glucosyl] $^+$) and *m/z* 287 ([M-glucosyl-glucosyl] $^+$), which corresponds to cyanidin diglucoside. Cmpd. 8 (Figure. 9B, t_R, 19.6 min) has molecular ion at *m/z* 449 and is fragmented to *m/z* 287 ([M-glucosyl] $^+$), which corresponds to cyanidin glucoside. The same identifications are applied to the derivatives of acetylglucoside and coumaroylglucoside. For example, Cmpd. 15 (Figure. 9C, t_R, 28.3 min) has molecular ion at *m/z* 491 and the fragment ion at *m/z* 287 ([M-acetylglucosyl] $^+$). Cmpd. 24 (Figure. 9E, t_R, 34.4 min) has molecular ion at *m/z* 595 and fragment ion at *m/z*

287 ([M-coumaroylglucosyl] $^{+}$). As such, they were identified as cyanidin acetylglucoside and cyanidin coumaroylglucoside, respectively. Cmpd. 30 (Figure. 9F, t_R, 23.2 min) with the molecular ion at *m/z* 561 and fragment ion at *m/z* 399 was identified as malvidin glucoside-pyruvate, formed through the interaction between malvidin glucoside and pyruvic acid. Cmpd. 31 (Figure. 9G, t_R, 26.2 min) and 32 (Figure. 9H, t_R, 32.0 min) have the same fragment ion at *m/z* 399, but different molecular ions at *m/z* 603 and at *m/z* 707 are assigned as mavidin acetylglucoside-pyruvate and malvidin coumaroulglucoside-pyruvate, respectively. Cmpd. 33 (Figure. 9I) has a molecule ion at *m/z* 609 and fragment ion at *m/z* 447, and is elucidated as malvidin glucoside-vinylphenol. The assignment for individual compounds agrees with previous report. [34-36]

By comparing the anthocynin profiles of fifteen grape juices (Table 3), most were found to exhibit similar anthocyanin compositions as Welch's Concord Grape Juice 100% concentrated (Figure. 8A), with the exception of Manischewitz Premium Grape Juice. In Manischewits Premium Grape Juice, only trace amount of delphinidin glucoside, cyanidin glucoside, petunidin acetylglucoside, and delphinidin coumaroylglucoside were detected. The major anthocyanins in the majority of the grape juices were cyanidin glucoside and delphinidin glucoside (Peak 6 and 7 in Figure. 8A). However, peonidin diglucose and malvidin diglucose dominate the anthocyanins in five out of fifteen grape juice samples (Sample # 2, 4, 8, 9, 12 in Table 1). The presence of the identified anthocyanins, as well as the other polyphenols in grape juices and other grape samples are shown in Table 3. (The chromatograms of each individual grape juice samples are shown in the Appendix I.)

Four different fresh grape berries were included to assess the differentiation of anthocyinin profiles, including one of the major red grape varieties, Cabernet Franc, a newly developed hydrid variety Noiret, and Red Seedless Table Grape and Black Seedless Table Grape from Pandol. Noiret was developed and named by Cornell University researchers working at the New York State Agricultural Experiment Stations, and was officially released on July 7, 2006. [42, 43] It is a hybrid variety with predominant ancestors *Vitis vinifera* and *Vitis labrusca* and has a black color and moderately large sized berries. [42, 43] Qualitative distribution of anthocyanins in four grape berries is quite different as shown in Table 3. Noiret Grape Berries contains highest variety of anthocyanin compounds, followed by Cabernet Franc Grape berries, Pandol Black Seedless Grape berries and Pandol Red Seedless Grape berries. The anthocyanin profile of Noiret Grape Berries is same as that in the Welch's Concerd Grape Juice 100% concentrated, with most abundant anthocyanin as delphinidin glucoside (Peak 6 in Figure. 8A). While the most abundant anthocyanin is malvidin glucoside (peak 11 in Figure. 8A) in Cabernet Franc Grape Berries and Pandol Red Seedless Grape Berries, and malvidin glucoside and malvidin coumaroylglucoside (Peak 11, 20 in Figure. 8A) in Pandol Black Seedless Grape Berries. The anthocyanin compositional difference between Noiret Grape Berries and other three berries is also characteristically distinguished by verifying the identities of the diglucoside derivatives (Table 3). In the Cabernet Franc Grape Berries, the diglucoside of anthocyanidins (Peak 1, 2, 3, 4, 5 in Figure. 8A) and coumaroylated derivatives of diglucosides (Peak 13a, 14a, 14b, 16a and 16b in Figure. 8A) were not detected. There are also no detectable diglucosides of anthocyanidins and coumaroylated

derivatives of diglucosides in the Pandol Red Seedless Grape Berries and Pandol Black Seedless Grape Berries (Table 3).

The qualitative distribution of anythocyanins between grape berries (whole fruit) and grape skins (peeled from the fresh fruit) is quite similar among the four analyzed grapes. But the anthocyanins are concentrationally dependent, with the higher concentration of anthocyanins in the skins. In this study, anthocyanins are asymmetrical distributed in the Cabernet Franc Grape Berries and Noiret Grape Berries. Subsequently, the grape skins from these two grape berries have an anthocyanin profile closer to that shown in the berries, but easily distinguished by each other by verifying the identities of the diglucoside derivatives as shown in Table 3 (MS spectrua of grape skins were shown in the Appendix I).

The color evolution of red wines is a complex process that is partially attributed to the progressive displacement of original anthocyanins by newly formed pigments. These pigments usually arise from the interaction between anthocyanins and other phenolic compounds, such as phenolic acid (pyruvic acid) and flavan-3-ols (catechins and procyanindins). In this report, Cabernet Franc Wine, Noriet Wine and Cabernet Sauvignon Wine were investigated to establish their anthocyanin profiles and furthermore to make a comparison. Overall in all three wines, we observed newly formed pigments among which malvidin glucose-pyruvate (Peak 24 in Figure. 8B) had the highest proportion. Cabernet Sauvignon Wine has a distinguished anthocyanin profile and most of them is malvidin relating compounds, including malvidin glucose and other newly

formed pigments, namely malvidin glucoside-pyruvate, malvidin acetylglucoside-pyruvate, malvidin coumaroylglucoside-pyruvate and malvidin glucoside-4-vinylphenol (Table 3). Only relatively low intensity of delphinidin glucoside-pyruvate and petunidin glucoside-pyruvate were identified in the Cabernet Sauvignon Wine. The distribution of anhocyanin profile for the Noiret Wine and Cabernet Franc Wine is close to those of their grape berries and corresponding grape skins (Table 3), only small amount were transferred to the newly formed pigment by interacting with other phenolic compounds in the samples. In three grape-derived dietary supplements (sample # 27-29 in Table 1), we observed glucoside derivatives and coumaroylglucoside derivatives of anthocyanidins, as well as trace amount of newly formed anthocyanin pigments.

Table 4. Presence of flavonols, phenolic acids, flavanols/proanthocyanidins in individual grape sample

Cmpd. Code	Sample Code																											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
34	+	+	+	+	+	+	+	T	+	+	+	+	+	+	+	T	T	+	+	+	+	-	-	-	+	+	T	T	+
35	+	+	+	+	-	+	+	-	T	+	+	T	+	+	+	+	+	+	+	+	T	T	-	-	+	+	-	-	-
36	+	+	+	+	+	+	+	T	+	+	+	+	+	+	+	+	T	T	+	+	-	-	+	+	+	+	T	T	+
37	+	+	+	+	+	+	+	T	+	+	+	+	+	+	+	+	+	+	+	+	+	T	+	+	+	+	+	+	+
38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-
41	+	+	+	+	T	+	+	T	+	+	+	+	+	+	+	T	T	+	+	+	+	+	-	-	T	T	T	T	T
42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
43	+	+	+	+	+	+	+	T	+	+	+	+	+	+	+	T	T	+	+	+	T	+	+	+	+	+	+	+	+
44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-
45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
46	+	T	+	+	T	+	+	-	T	T	T	T	T	T	T	T	T	T	T	T	T	+	-	-	-	-	+	+	+
47	+	+	+	+	+	+	+	T	+	+	+	-	+	+	+	T	T	T	-	-	-	+	+	+	+	+	+	+	+
48	+	+	+	+	T	+	+	-	-	+	+	+	+	+	+	T	T	T	-	-	+	+	-	-	-	-	+	+	+
49	T	-	-	-	-	-	-	-	-	-	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50	+	+	+	+	T	+	+	-	-	+	+	T	T	-	+	-	-	T	-	-	T	+	-	-	-	-	-	-	-
51	+	+	+	+	T	+	+	-	-	+	+	-	-	+	+	-	-	+	-	-	+	+	-	-	-	-	+	+	+
52	T	T	T	+	T	+	+	-	-	+	+	T	T	T	+	-	-	+	-	-	+	+	-	-	-	-	-	-	-
53	+	+	+	+	T	+	+	-	-	+	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-	-
Total	14	13	13	13	12	13	13	6	8	13	14	12	11	11	13	9	9	13	7	7	11	17	4	4	7	7	9	9	9

Sample codes are same as in Table 1. Compound codes refer to Table 2. (+) Present, (-) not detectable, T: trace, Total: total number of flavonols, phenolic acids, flavanols/proanthocyanidins present in individual samples.

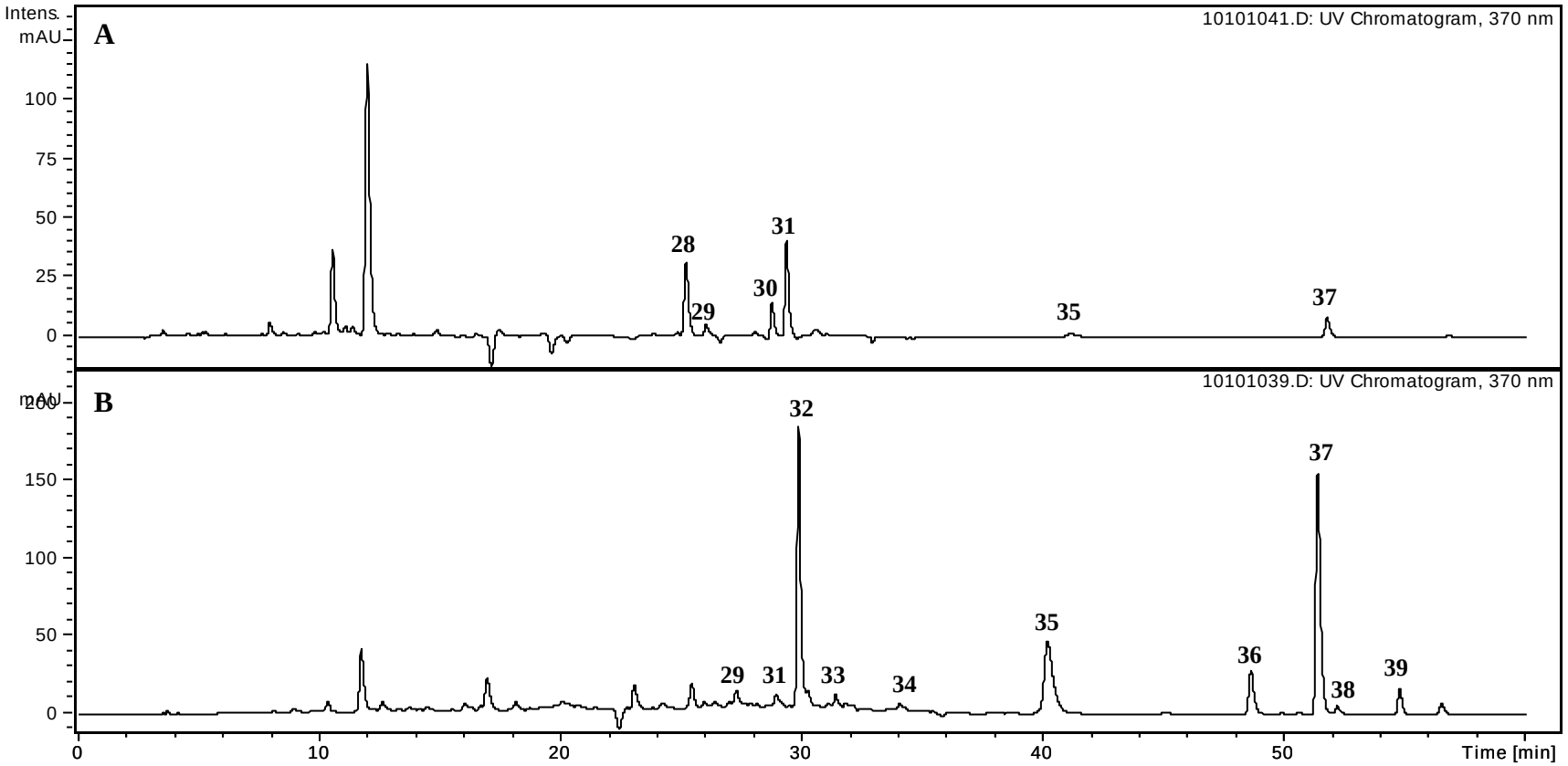

Figure 11. Representative UV chromatograms (370 nm) of (A) Welch's Concord Grape Juice 100% concentrated and (B) Cabernet Sauvignon Wine. Peak assignment, t_R, identities are listed in Table 2. The presence of flavonol profile for each individual samples is listed in Table 4.

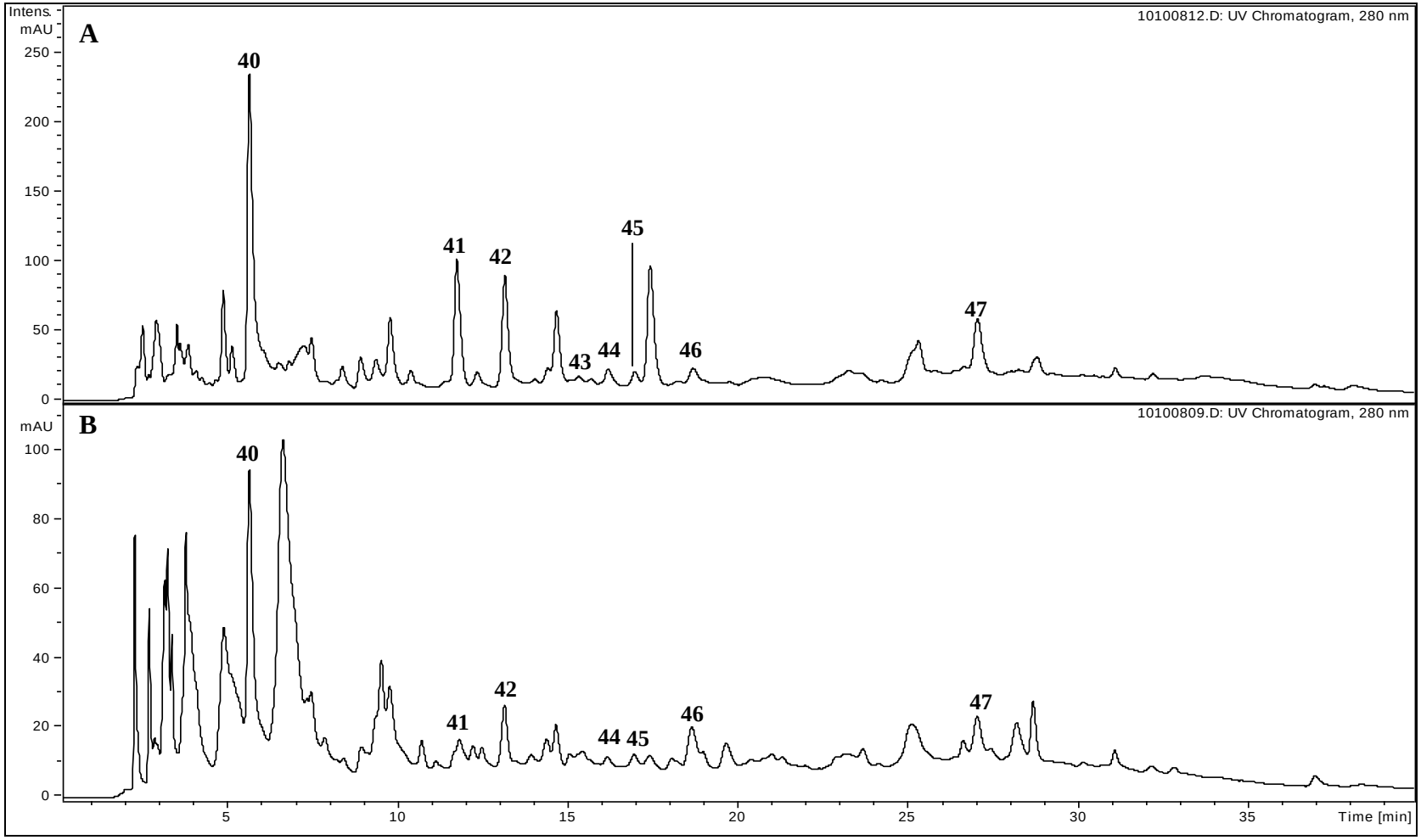

Figure 12. Representative UV chromatograms (280 nm) of (A) Welch's Concord Grape Juice 100% concentrated and (B) Cabernet Sauvignon Wine. Peak assignment, t_R, identities are listed in Table 2. The presence of phenolic acid and flavanol/proanthocyandin profile nfor each individual samples is listed in Table 4.

2.4.2 Flavonol identification and profile comparison

By comparing the flavonol profiles of fifteen grape juice samples at UV wavelength at 370 nm, we observed that they have very similar flavonol composition as Welch's Concord grape juice 100% concentrated (Figure.11A and Table 4). The major free flavonols aglycone in the various commercial grape juices are myricetin and quercetin, which are at 41.0 min and 51.6 min in Figure 11A. While their glucoside or glucuronide were eluted much earlier due to the addition of very hydrophilic molecular glucose and glucoronic acid. Cmpd. 34 (t_R, 25.2 min) has the molecular ion at *m/z* 481 and fragment ion at *m/z* 319 ([M-glucosyl] $^+$) and therefore, is assigned as myricetin glucoside. Cmpd. 35 also has the fragment ion at *m/z* 319, but it is produced from molecular ion at *m/z* 495 by losing 176 which is usually from glucuronide, and is identified as myricetin glucuronide. Same identification could be achieved for quercetin glucoside and quercetin glucuronide.

Qualitative distribution of flavonols in Cabernet Franc Grape Berries and Skins, Noiret Grape Berries and Skins and Pandol Black Seedless Grape Berries and Skins are similar as shown in Table 4. Majority of the flavonols in the 4 original grape samples are myricetin glucoside, myricetin glucuronide, quercetin glucoside and quercetin glucuronide. In Pandal Red Seedless Grape Berries and Skins only quercetin glucoside and quercetin glucuronide were detected. In this report, Cabernet Franc wine, Noiret Wine and Cabernet Sauvignon Wine were investigated to establish their flavonol profiles and to make a comparison. Overall in all the three wine samples, Cabernet Franc Wine and Noriet Wine show the similar profile as that of the corresponding grape berries and

skins, and grape juice products, with the major flavonals of either aglycons (myricetin and quercetin) or the corresponding glucoside and glucuronide. But in Cabernet Sauvignon Wine, the flavonol profile is significantly different from the grape juice products, Cabernet Franc Wine and Noiret wine (Table 4). Syringetin glucoside (Peak 32) is the dominant flavonol in the Cabernet Sauvignon Wine as shown in Figure 11B. Othe flavonols, such as syringetin acetylglucoside, isorhamnetin glucuronide, syringetin, laricitrin and isorhamnetin (Peak 33, 34, 36, 38, 39) is only detected in the Cabernet Sauvignon Wine (Figure. 11B). Additionally, myricetin glucoside and quercetin glucoside (Peak 28 and 30) were not detected in the Cabernet Sauvignon Wine (Figure. 11B).

2.4.3 Phenolic acid and flavanol identification and profile comparison

The detection of phenolic acid and flavanols were achieved under the negative mode with the same LC gradient as the anthocyanins and flavonols. On the basis of UV and MS spectral data and by comparing to the retention time of the purchased standards, we were able to detect 4 phenolic acids, catechin, *epi*-catechin and proanthocyanidin dimmers. Thirteen out of fifteen grape juices exhibited similar profile of phenolic acid and proanthocyanins. In the Shoppers Value Grape Drink, trace amount of proanthocyanidin dimer was identified. In ACME Grape Juice Cocktail, only gallic acid and proanthocyanidin dimer were detected. Grape berries and their corresponding grape skins contain same composition of phenolic acids, while in their corresponding wine samples, more phenolic acids were found (Table 4). The representative UV chromatograms (280

nm) of Welch's Concord Grape Juice 100% concentrated and Cabernet Sauvignon Wine are illustrated in Figure 11.

2.5 CONCLUSIONS

A rapid and comprehensive qualitative method was developed to characterize the polyphenols, such as anthocyanins, flavonols, phenolic acids and flavanols/proanthocyanidins, in grapes and grape-derived products. The detection was achieved by two runs with same LC gradient, but different MS ionization modes and mobile phase modifers. Anthocyanins and flavonols were detected under positive ion mode with 0.4% trifluoroacetic acid (v/v) in water and acetonitrile, phenolic acids and flavanols were detected under negative mode with 0.1% formic acid (v/v) in water and in acetonitrile. Under the optimized LC/MS conditions and on the basis of analizing the MS and UV data and in comparion with the authenticated standards, a total of 53 compounds were identified, including 33 anthocyanins, 12 flavonols, 4 phenolic acids and 4 flavanols. With the method developed, 15 grape juices, 4 grape berries and skins, 3 wines and 3 grape-derived dietary supplemnts were qualitatively investigated to assess and compare the composition of polyphenols among them. This method provided complete insight into the composition of polyphenols in grapes, grape juices, wines and other grape-derived products. It can be used for the control of new grape-related products' quality, and evaluation of parameters affecting the polyphenol compositions and accumulations during the production, harvest and storage.

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CHAPTER 3

QUANTIFICAITON OF ANTHOCYANIDINS IN THE GRAPES AND GRAPE JUICE PRODUCTS WITH ACID ASSISTED HYDROLYSIS USING LC/MS

3.1 ABSTRACT

A simple and precise acid assisted hydrolysis method was established for the quantitation of anthocyanidins in 15 grape juice samples, 4 grape berries and 4 grape skins using LC/MS. Under optimized conditions, five major anthocyanidins including delphinidin, cyanidin, petunidin, peonidin and malvidin in the hydrolyzed grape extracts were successfully separated within 25 min and quantitated individually. The results revealed that the total concentration of anthocyanidins was not symmetrically distributed in the various brands of grape. Rather, among all fifteen grape juices, peonidin was found to be present in lowest concentration. The quantitative distribution of anthocyanidins in grape berries and skins are quite similar, although anthocyanidin concentration in grape skins is four to eight times higher than their corresponding berries. The precision of this method was validated by recovery percentages of five anthocyanidins, ranging from 98.59 % to 103.20% with the relative standard deviation (RSD) less than 5.03%. This quantitative method provides a rapid and accurate tool to quantitatively study individual anthocyanidins in grapes or grape juice samples for quality control and to facilitate the evaluation and comparison of new commercial grapes or grape juices products in market.

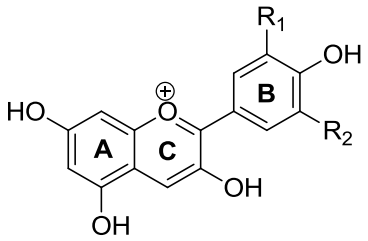
3.2 INTRODUCTION

Anthocyanins are versatile red, blue or purple plant pigments that are widely distributed in plant tissues, mostly in flowers, fruits and vegetable, but also in leaves, stems and roots. [1] Dietary comsumption of foods and products enrich in anthocyanins has become increasingly popular due to their beneficial health effects. As natural colorants, they are non-toxic, water soluble and very easy to incorporate in the aqueous media, which makes them good candidates to substitute synthetic colorants that have shown to exhibit toxicity in humans. [6, 7] Another extensive and attractive property of anthocyanins is due to their antioxidant capacity which has been exhaustively studied. [8-14] Reactive oxygen species such as hydroxyl, peroxyl and superoxide anion radicals and reactive nitrogen species such as nitric oxide are constantly generated in animals and humans from metabolic reactions. Extreme radical production may surpass the antioxidant capacity provided by endogenous antioxidant enzymes and compounds such as superoxide dismutase, glutathione peroxidase and glutathione. Consequently it may induce the damage and dysfunction of genetic materials and cell membranes by attacking proteins, lipids and DNA. [15-18] Anthocyanins can scavenge extra radical ions and release the oxidation stress, therefore the antioxidant activity of anthocyanins potentially contributes to the prevention of various diseases, such as neuronal and cardiovascular illness, cancer and diabetes. Anthocyanins were shown to aid in the prevention of heart disease, especially in the form of grape juice and wine, but also from many other plant-based sources. Epidemiological studies have shown that coronary heart disease mortality can be decreased by moderate consumption of red wine. [19, 20] A study on the relationshops between vasodilation capacity, antioxidant activity and phenolic contect of 16 red wines reported that the total

phenol content was correlated very closely with the antioxidant activity and vasodilation activity, but only the total anthocyanins were correlated with vasodilation activity. [21] Anthocyanins were shown to help in the prevention of cancers. [22-23] For example, bilberry extracts enriched in anthocyanins inhibited the growth of HL60 cells through the induction of apoptosis. [24] Anthocyanins were also reported to the treatment and prevention of diabetes. A study on exploring the effects of anthocyanins and anthocyanidins on the insulin secretion reported that anthocyanins and anthocyanidins stimulated insulin secretion when exposed to pancreatic β-cell, which was potentially useful for the treatment of type 2 diabetes. [25]

Anthocyanidin, the aglycone of anthocyanins, possess the typical $C_6C_3C_6$ carbon skeleton of flavonoids. And they are distinguished from other flavonoids as a separate class by virtue of their ability to form flavylium cations. Individual anthocyanidins are differentiated by the number of hydroxyl group and the degree of methylation on the flavylium salts. As a glycoside of anthocyanidins, anthocyanins are attached with one or more different sugars, such as glucose, galactose, rhamnose, arabinose, xylose and glucuronic acid at most often C-3, C-5, or C-7 positions. [5] In addition, diversity is further increased by the chemical combination of sugars with aromatic or aliphatic acid, such as acetic, succinic, caffeic acid and many more to produce acylated anthocyanins. [5] Considering all these factors, the variety and number of anthocyanins is large, with an estimated 600 individual anthocyanins identified in the nature. [26-28, 5] The wide variety of anthocyanins coupled with their often chemical similarity results in great number of peaks on chromatograms and difficulty in separating and indentifying individual

anthocyanins in the grape matrices. In our previous qualitative study (in Chapter 2), we identified a total of 33 anthocyanins, in which 27 anthocyanins were once detected in a single grape juice sample. Given the enormous variety of anthocyanins, only a few reference compounds are commercially available, making it difficult to establish a definitive method to accurately quantify the anthocyanin composition and concentration in the grape and grape juice products. Scientists have been estimated the anthocyanins by choosing one reference glycoside for calibrations based on the fact that the chromatographic response of anthocyanins was very similar at 520 nm. [29] This method has the limitation in that can only be used when all the anthocyanins in the sample can be fully separated on a single based line. Otherwise the interference between inspeparable peaks will either overestimate or underestimate the absolute quantities of the compounds. Fortunately, the various anthocyanin glycoside patterns can be brought down to 23 [2] naturally occurring anthocyanidins, in which six most common anthocyanidins include delphinidin, cyanidin, petunidin, peonidin, pelargonidin and malvidin as shown in Figure 13. [5] Therefore, the problem can be overcomed by establishing a reliable quantification method that identifies or reveals the compositions of anthocynidins, the aglycone forms of anthocyanins.



Anthocyanidin	Abbreviation	R_1	R_2
Cyanidin	Cy	OH	H
Delphinidin	Dp	OH	OH
Malvidin	Mv	OCH_3	OCH_3
Pelargonidin	Pg	H	H
Peonidin	Pn	OCH_3	H
Petunidin	Pt	OH	OCH_3

Figure 13. General structure of six most common anthocyanidins

Furthermore, different anthocyanidins have significantly different bioavailability, physiological and functional properties. [30] Thus in order to leverage the best benefit from them, information on both chemical structures and quantity of individual anthocyanidin in grapes and grape juices is essential. In addition, the aglycones, as a sugar free compartment in the plant, are of more biological interest than their conjugated forms. In some plants the conjugated forms are utilized to transport and store the less soluble aglycones. Upon microbial infections, the conjugated forms are metabolized to aglycone to exhibit the biological activities in humans and animals. [31-35] In the structure and antioxidant activity relationship study, researchers found that aglycones are more effective than corresponding glycoside, probably caused by its extra chelation site or its increased lipophilicity. Glycosylation of hydroxyl group might mask the antioxidant activity. [36]

Given the significance to evaluate the quantity of anthocyanidins (aglycone form) and feasibility to quantify the antocyanidins, the objective of this work was to develop a simple, rapid and accurate acidic hydrolysis method to quantitatively evaluate the individual anthocyanidin compostion in grapes and grape juices. Further more, using the method developed, a range of commercial retail grape juices as well as four grape berries and grape skins were included to determine the composition of anthocyanidins between the products. Then the method would be validated by spiking known amont of anthocyanidins in represented grape juice sample to evaluate the the recovery. The same procedure would be repeated in three differenct day to check and repeatability.

3.3 MATERIALS AND METHODS

3.3.1 Materials

Anthocyanidin standard compounds were purchased from ChromaDex (Irvine, CA) and used as received. The HPLC purities were cyanidin chloride 97.4%, delphinidin chloride 97.6%, malvidin chloride 93.2%, petunidin chloride 98.5% and peonidin chloride 99.5%. HPLC grade acetonitrile (ACN), methanol (MeOH), trifluoroacetic acid (TFA) were obtained from Fisher Scientific Co. (Fair Lawn, NJ). HPLC grade formic acid was obtained from Acros Organics (NJ). HPLC-grade water (18MΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corporation, Bedford, MA). Fifteen retail grape juices were purchased from several food super markets and they covered various brands, such as Welch's®, Langers®, Healthy Balance®, Wild Harvest®, Shopper Value®, ACME®, ShopRite®, Santa Cruz®, Snapple®, Walgreens®, Manischewitz® and Kedem® as shown in Table 5. Welch's Concord Grape Juice 100% concentrated,

Cabernet Franc Grape Berries and Noriet Grape Berries as a whole fruit were provided by Purdue University. Pandal Red/Rlack Seedless Table Grape were commericially purchased. The grape skins were manually peeled from fresh grape berries. All grape juices were stored at 4 ℃ and grapes/skins were stored in -20 ℃.

T**able 5.** Information of experimental samples of grapes and grape-derived products

Sample code	Sample name	Internal QC Ref. Num.	Original source
1	Welch's Concord grape juice 100% concentrated	BC/0902200023	Purdue Univ.
2	Welch's concord grape juice cocktail	NAU1010010002	ACME
3	Welch's light concord grape juice beverage	NAU1010010003	ACME
4	Welch's 100% juice black cherry concord grape juice	NAU1010010004	ACME
5	Langers pomegranate grape juice	NAU1010010006	ACME
6	Healthy Balance grape juice	NAU1010010007	ACME
7	Wild Harvest organic grape juice	NAU1010010008	ACME
8	Shoppers Value grape drink	NAU1010010009	ACME
9	ACME grape juice cocktail	NAU1010010010	ACME
10	ShopRite pasteurized grape juice	NAU1010010011	ShopRite
11	Santa Cruz organic concord grape juice	NAU1010010012	ShopRite
12	Snapple naturally flavored grapeade juice	NAU1010010013	ShopRite
13	Walgreens Grape Juice	NAU1010040014	Walgreens
14	Manischewitz premium grape juice	NAU1010060015	A&P
15	Kedem concord grape juice	NAU1010060016	A&P
16	Cabernet Franc grape berries	BC/0810140001	Purdue Univ.
17	Cabernet Franc grape skins	BC/0810140002	Purdue Univ.
18	Noiret grape berries	BC/0810140005	Purdue Univ.
19	Noiret grape skins	BC/0810140006	Purdue Univ.
20	Pandol red seedless table grape berries	NAU1010090017	Costco
21	Pandol red seedless table grape skins	NAU1010090018	Costco
22	Pandol black seedless table grape berries	NAU1010090019	Costco
23	Pandol black seedless table grape skins	NAU1010090020	Costco

3.3.2 Sample Preparations

For quantitative determination, grape juices (2.5 mL) were dispersed in 2.5 mL MeOH and 1.5 mL concentrated HCl (12 M) to make the final solution as 2.7 M HCl in MeOH, and transferred into a 20 mL glass bottle for hydrolysis. The glass bottles were capped and put into 90℃ water bath for 60 min. Then the cooled sample was decanted into 10mL volumetric flask and brought up to the final volume of 10ml by adding methanol to keep the volume constant. The grape berries as a whole fruit were frozen fully in – 20 ℃ and then grounded into small pieces. Grape skins were manually peeled from fresh grape berries and then grouded. Around 200 mg of grape berrie and around 100 mg grape skin were dispersed in 5 mL 2.7 M HCl (23 mL 37% HCl + 77 mL MeOH) and transferred into a 20 mL glass bottle for hydrolysis. The glass bottles were capped and put into 90 ℃ water bath for 60 min. Then the cooled sample was decanted into 25 mL volumetric flask and brought up to the final volume of 25 ml by add methanol. All samples were filtered into HPLC vials through 0.45 um filter prior to the injection. The recoveries were validated by spiking known quantities of standard compounds, dephinidin, petunidin, cyanidin, malvidin and peonidin, to approximately 100, 75, and 50% of the expected values in the Welch's Concord Grape Juice 100% concentrated and then hydrolyzing using the same procedure developed.

3.3.3 Equipments and HPLC-MS conditions

HPLC separation was performed on a Polaris amide-C18 column, 250 x 4.6mm, 5 μM (Varian Inc.). For LC-MS analysis, a Hewlett Packard Agilent 1100 Series LC/MS (Agilent Technologies, Waldbronn, Germany) equipped with quaternary pump system,

diode array and multiple wavelength detector, degrasser, MSD trap with an electrospray ion source (ESI), and software of HP ChemStation, Bruker Daltonics 4.2 and Data Analysis 4.2 was used. The mobile phase containing solvent A and B in gradient, where A is 0.4% TFA (v/v) in water and B is 0.4 % TFA (v/v) in acetonitrile for the following gradient: 0-5 min, isocratic elution at 25 % B; 5-20 min, linear gradient from 25% to 40% B; 20-25 min, linear gradient from 40% to 50%. The flow rate was set at 1.0 mL/min. The injection volume was 10 μL and the detector was set at 254, 280, 370, 520 nm. The eluent was monitored by electrospray ion mass spectrometer (ESI-SIM) under positive ionization mode scanned from *m/z* 100 to 900. Under SIM (selected ion monitoring), molecular ions $[M]^+$ were isolated for each individual analytes. The mass spectrometer was set into three segments: (1) from 0 to 10 min for delphinidin with isolation of *m/z* 303, (2) from 10 to 15 min for petunidin and cyanidin of *m/z* 317 and 287 and (3) from 15 to 25 min for malvidin and peonidin of *m/z* 331 and 301 . The isolation width was set as 1.0 *m/z*. ESI was conducted by using a needle voltage of 3.5KV under optimum collision energy level of 60%. High-purity nitrogen (99.999%) was used as dry gas and at a flow rate of 12 L/min capillary temperature at 350 ^{o}C. Nitrogen was used as nebulizer at 60 psi and Helium as collision gas. Identification of dephinidin, petunidin, cyanidin, malvidin and peonidin was based on the LC/MS data as well as in comparison with the authenticated standards.

3.3.4 Standard and calibration curve

Each standard stock solution was prepared by dissolving the appropriate amounts of ~2.0 mg commercially available reference anthocyanidins in 10 mL methanol solution

containing 2.7 M HCl (23mL 37% HCl + 77mL MeOH). Each standard stock solution was sonicated for 10 min, and was allowed to cool down to room temperature. 2 mL of each standard stock solution was combined together and sonicated for 10 min to mix well to form a standard mixture of delphinidin, petunidin, cyanidin, malvidin and peonidin. The calibration curves were established on 12 data points by diluting the standard mixture with methanol solution containing 2.7 M HCl to cover the expected concentration range of samples. Calibration curves were plotted using peak areas of UV absorption at 520 nm versus the concentration in μmol/L. The calibration concentrations ranged from 0.065 μmol/L to 265.722 μmol/L with equation $y = 14.778x - 10.91$ (r^2 = 0.9998) for depinidin, from 0.101 μmol/L to 207.908 μmol/L with equation $y = 4.2449x - 1.1358$ ($r^2 = 1$) for petunidin, from 0.047 μmol/L to 387.357 μmol/L with equation $y = 13.133x - 4.8558$ ($r^2 = 1$) for cyanidin, from 0.074 μmol/L to 18.935 μmol/L with equation $y = 12.696x + 3.9252$ (r^2 =0.9951) for malvidin, and from 0.036 μmol/L ~296.983 μmol/L with equation $y = 17.961x - 0.2986$ ($r^2 = 1$) for peonidin.

3.4 RESULTS AND DISCUSSIONS

3.4.1 Method optimization

Anthocyanins undergo transformations with changes in pH, which has a dramatic effect on color. [5, 37-38] In aqueous solutions, at pH value approximately 3 or lower, [5] flavylium cation is the predominant species and contributes to the orange and red color. As the pH increased, kinetic and thermodynamic competition occurs between the hydration of the flavylium cation and proton transfer from its acidic hydroxyl group. [5] In the former reaction, the anthocyanin molecular turns into colorless carbinol pseudo-base, which can

undergo ring opening to a yellow chalcone. The latter reactions give rise to quinonoidal base, then quinonoid anions. [5] Since the anthocyanidin system undergoes a variety of molecular transformations as the pH changes, it complicates the spectrometric detection, compounds separation on the column, and peak shape is greatly affected by the pH of the samples and mobile phase modifier for HPLC seperation. Therefore, in order to decrease the possibility of anthocyanidin structure transformations along the pH value and to maintain high degree of recovery in the hydrolysis study, we tested a series of HCl acid concentration (1.0 M, 2.7M, 4.0M and 6.0 M). The result shown that promising condition was 2.7 M HCl in the 90℃ water bath for 60 min. In this condition, it assured the anthocyanidin flavylium cations as the dominant structure in the solution, and at the same time maintained the high recovery yield. The matrix of anthocyanidins standard solutions should resemble that of samples in the hydrolysis studies. Therefore, 2.7 M HCl in the methanol was used to dissolve the anythocyanidins standards. 0.4% TFA was selected as the mobile phase modifier in the aqueous and organic eluting solvent to narrow down the polarity range of anthocyanidins on the column.

3.4.2 Quantitative survey of anthocyanidins in different grape juice products and grape berries and skins

Previous qualitative study (Charpter 2) enabled us to indentify various anthocyanins in the grape juices, grape berries and grape skins. Due to the high numbers of different anthocyanins and limited availability of commercial standards, it was difficult to develop an analytical method to accurately quantitate all the anthocyanins in original grape samples. To facilitate the quantification and accurately evaluate the total anthocyanidins

in the grape juices and grape berries and grape skins, we developed an acid assisted hydrolysis method. In our previous study we only detected trace amout of pelargonidin derivatives that can not be detected in all grape juice samples (Chapter 2), therefore, pelargonidin was not included in the total anthocyanidins in this quantification study. Under optimized LC-MS condition, five anthocyanidins, delphinidin, cyanidin, petunidin, peonidin and malvidin were successfully separated within 25 minutes and quantified in the hydrolyzed samples by UV detection at 520 nm. Baseline separation was successfully achieved as shown in Figure 14.

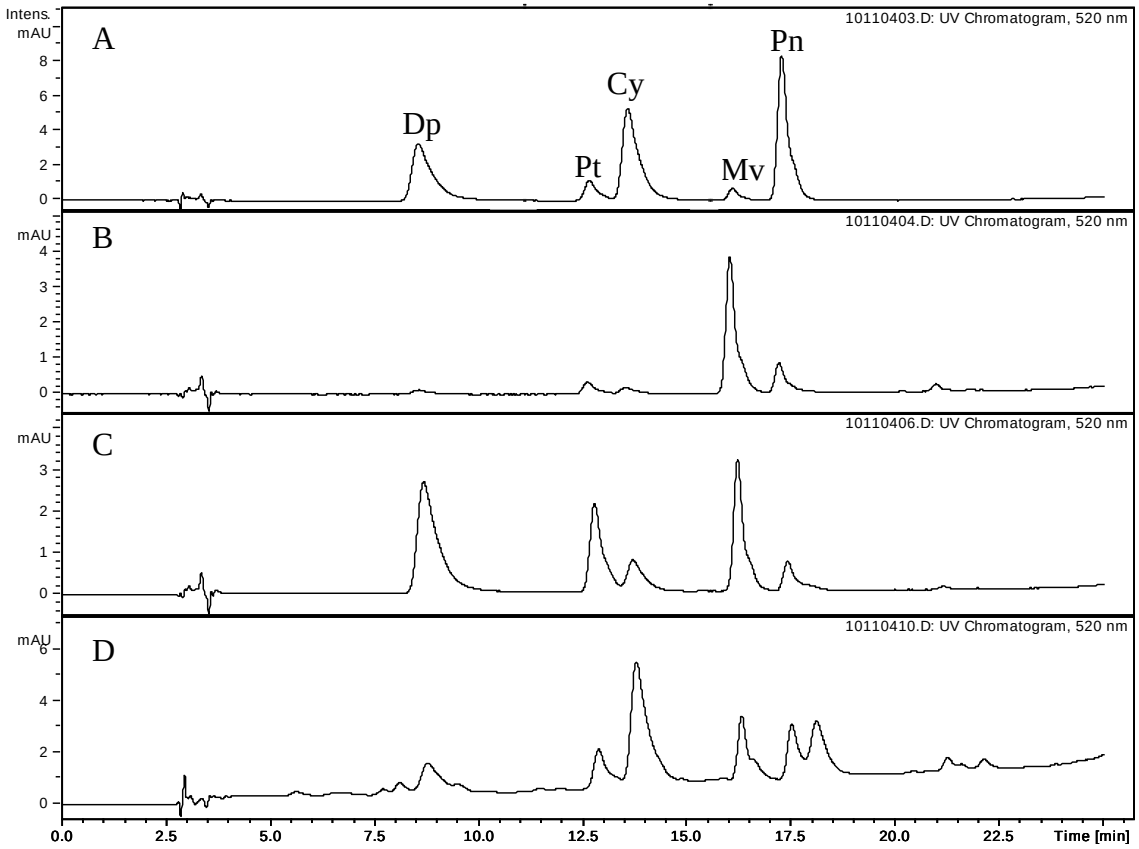


Figure 14. UV chromatograms (520 nm) of (A) standards mixture of Dp (delphinidin), Pt (petunidin), Cy (cyanidin) , Mv (malvidin) and Pn (peonidin), and representative

hydrolyzed samples (B) Cabernet Franc grape berries, (C) Noiret grape berries, (D) Welch's Concord grape juice 100% concentrated

Utilizing the method developed above, we surveyed the distributions of anthocyanidins in a series of grape juice samples with various brands. Concentrations of each of the anthocyanidins were expressed as μmol per L for grape juice samples and the total concentration of anthocyanidins was calculated by adding up the individual concentration of five anthocyanidins (Table 6 and Figure. 15). The total concentrations of anthocyanidins are not symmetrically distributed in the various brands of grape juices. Welch's concord grape juice 100% concentrated contained highest total concentration of anthocyanidins (161.59 μmol/L) among the grape juices we surveyed, followed by Santa Cruz organic concord grape juice (91.92 μmol/L), in which the total concentration of anthocyanidins is about one half of that in Welch's concord grape juice 100% concentrated. Four out of fifteen grape juices fallen into the category of total concentration of anthocyanidins between 60 to 80 μmol/L, including Kedem concord grape juice (79.98 μmol/L), Welch's 100% juice black cherry concord grape juice (73.97 μmol/L), Wild Harvest organic grape juice (61.42 μmol/L) and Welch's light concord grape juice beverage (60.32 μmol/L). The total concentrations of anthocyanidins in the rest of grape juices were from 10 to 40 μmol/L. The dominant anthocyanidin is not consistent across all grape juices samples we surveyed. Cyanidin is the most concentrated anthocyanidin in seven out of fifteen grape juices (Sample # 1, 3, 5, 7, 11, 13, 14). Malvidin is the most concentrated anthocyanidin in five out of fifteen grape juices (Sample # 2, 4, 8, 9, 12). In the other three grape juices (Sample # 6, 10, 15), petunidin has the highest concentration among five anthocyanidins. Interestingly, in all fifteen

grape juices we sampled, we observed that peonidin was consistently present in the lowest concentration, ranging from 0.42 μmol/L in Manischewitz premium grape juice to 9.97 μmol/L in Welch's Concord Grape Juice 100% concentrated.

Table 6. Concentration of anthocyanidins in grape juices

Grape juice samples (µmol/L)						
Sample code	Dp	Pt	Cy	Mv	Pn	Total
1	33.45 ±0.18	42.56 ±0.63	55.44 ±0.48	20.17 ±0.12	9.97 ±0.03	161.59 ±1.44
2	5.35 ±0.06	8.07 ±0.19	5.31 ±0.09	14.69 ±0.27	4.59 ±0.13	38.02 ±0.75
3	13.98 ±0.16	15.93 ±0.10	23.36 ±0.23	4.89 ±0.11	2.17 ±0.02	60.32 ±0.62
4	9.79 ±0.03	17.44 ±0.12	11.37 ±0.10	26.32 ±0.04	9.06 ±0.03	73.97 ±0.32
5	5.50 ±0.03	5.75 ±0.12	13.45 ±0.10	8.57 ±0.04	2.42 ±0.03	35.69 ±0.32
6	10.63 ±0.12	18.00 ±0.36	10.74 ±0.17	8.12 ±0.26	3.25 ±0.07	50.74 ±0.98
7	12.24 ±0.13	18.09 ±0.09	18.96 ±0.16	8.76 ±0.03	3.37 ±0.03	61.42 ±0.44
8	3.14 ±0.01	2.55 ±0.03	2.39 ±0.01	4.34 ±0.09	1.70 ±0.09	14.11 ±0.24
9	4.23 ±0.03	3.99 ±0.02	4.34 ±0.02	4.37 ±0.02	1.84 ±0.02	18.78 ±0.11
10	8.56 ±0.06	9.99 ±0.14	9.63 ±0.07	7.52 ±0.02	2.29 ±0.02	38.00 ±0.30
11	20.74 ±0.23	25.63 ±0.46	30.46 ±0.28	10.52 ±0.03	4.57 ±0.09	91.92 ±1.09
12	3.21 ±0.01	3.99 ±0.08	2.95 ±0.02	7.99 ±0.10	2.35 ±0.03	20.50 ±0.24

13	6.43 ± 0.04	6.44 ± 0.07	6.71 ± 0.03	5.05 ± 0.09	1.15 ± 0.01	25.78 ± 0.25
14	3.70 ± 0.04	4.21 ± 0.10	4.67 ± 0.04	3.26 ± 0.16	0.42 ± 0.02	16.25 ± 0.35
15	14.26 ± 0.61	24.38 ± 0.88	17.76 ± 0.57	17.39 ± 0.42	6.19 ± 0.17	79.98 ± 2.65
Grape berries and grape skins (mg/kg)						
Sample code	Dp	Pt	Cy	Mv	Pn	Total
16	51.83 ± 3.90	142.46 ± 1.37	29.29 ± 2.53	335.33 ± 27.68	48.68 ± 4.11	607.60 ± 39.60
17	181.82 ± 7.27	486.59 ± 37.94	133.66 ± 19.60	1380.22 ± 44.87	177.21 ± 15.21	2359.51 ± 124.89
18	483.04 ± 11.03	861.08 ± 42.07	109.90 ± 8.05	333.52 ± 14.27	50.16 ± 2.14	1837.70 ± 77.56
19	4148.45 ± 261.72	6941.57 ± 359.33	742.21 ± 42.92	2403.70 ± 57.02	372.45 ± 5.07	14608.38 ± 726.06
20	6.59 ± 0.15	5.04 ± 0.34	18.48 ± 0.75	0.0033 ± 0.0001	12.18 ± 1.29	42.30 ± 2.53
21	16.76 ± 0.33	17.78 ± 0.45	94.06 ± 0.32	0.32 ± 0.28	76.83 ± 3.17	205.75 ± 4.54
22	33.55 ± 3.11	78.68 ± 6.77	28.94 ± 2.12	215.36 ± 21.49	13.37 ± 1.17	369.90 ± 34.66
23	159.89 ± 4.86	568.60 ± 13.24	199.91 ± 17.04	1668.20 ± 125.77	155.23 ± 12.81	2751.83 ± 173.73

Values represent means of triplicate determinations across three different days (n=3) ± SD. Sample codes are same as in Table 5. Concentration is expressed as μmol L^{-1} for grape juice samples and is expressed as mg kg^{-1} for grape berries/skins. Dp (delphinidin), Pt (petunidin), Cy (cyanidin), Mv (malvidin) and Pn (peonidin).

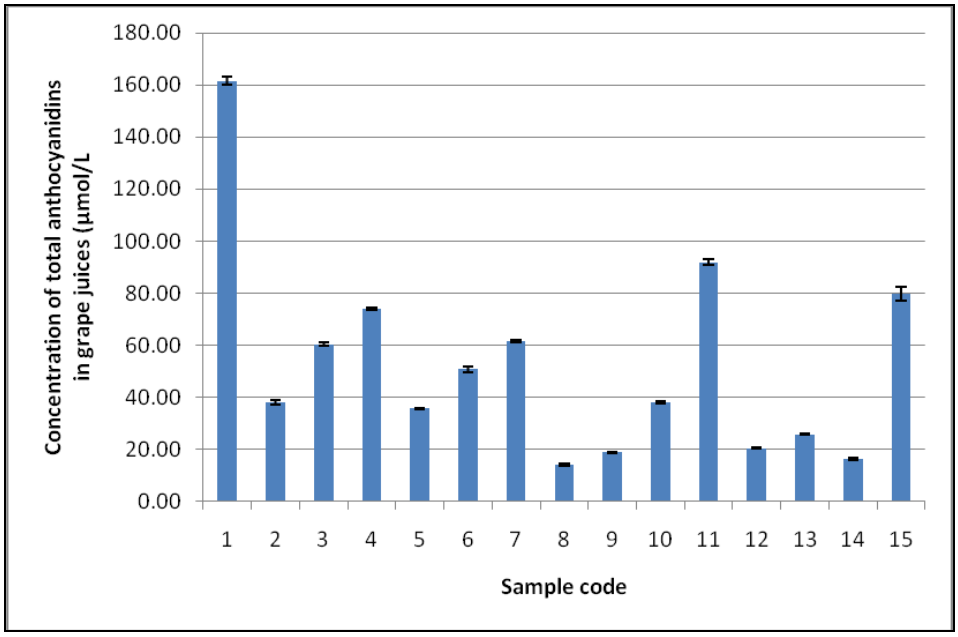


Figure 15. Concentration of total anthocyanidins in grape juices. Values represent means of triplicate determinations (n=3) ± SD. Sample codes are as in Table 5.

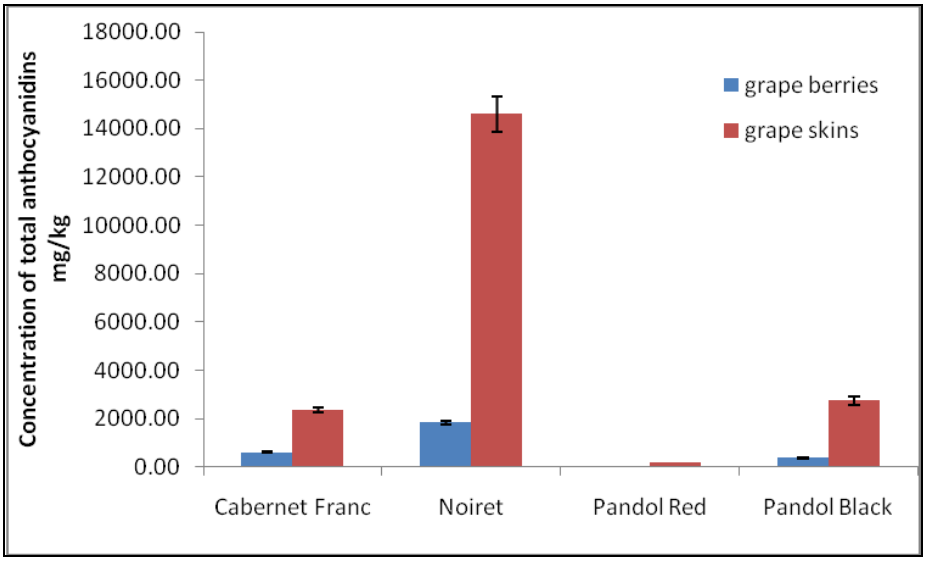


Figure 16. Comparison of total anthocyanidins in grape berries and skins. Values represent means of triplicate determinations (n=3) ± SD. Sample codes are as in Table 5.

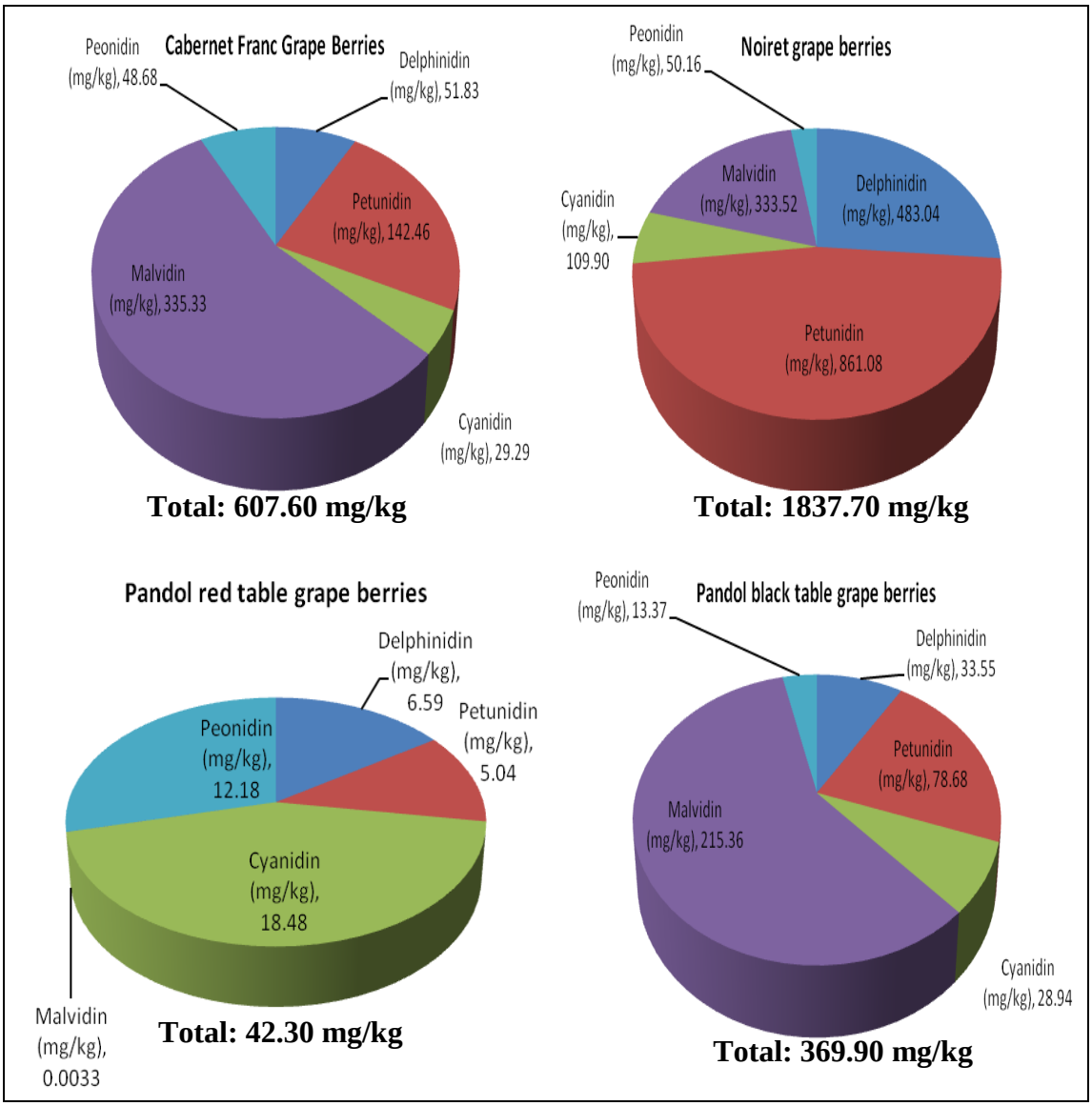


Figure 17. Comparison of anthocyanidin compositions in four grape berries

Different varieties of grape berries and grape skins were included to compare and contrast the composition of five anthocyanidins in the different source. Cabernet Franc and Noiret Grapes were provided by Purdue University and Pandol red/black seedless table grapes were commercially purchased as fresh berries. Grape skin was peeled from fresh grape berry manually. Cabernet Franc is one of the major red grape varieties worldwide. It can either be processed alone to make wines or it can be blended with Cabernet Sauvignon and Merlot in the Bordeaux style. Noiret was a newly developed hydrid variety by Cornell University officially released in 2006. Noiret has its predominant ancestors *Vitis vinifera* and *Vitis labrusca* background, with a black and relatively large-sized berries. [39-40] The concentrations of each anthocyanidins in grape berries and skins are expressed as mg per kg fresh grape berries as shown in Table 6. Overall, the anthocyanins in grape berries and skins are concentrationally dependent, with the higher concentration of anthocyanins in grape skins. The concentration of anthocyanidins in grape skins is four to eight times higher than that in their corresponding berries. The total concentration of anthocyanidins in four grapes is different as shown in Table 6. Noiret Grape Berries contained the highest total concentration of anthocyanidins (1837.70 mg/kg), followed by Cabernet Franc Grape Berries (607.60 mg/kg), Pandol Black Seedless Grape Berries (369.90 mg/kg) and Pandol Red Seedless Grape Berries (42.30 mg/kg). The dominant anthocyanidin in each type of grapes is quite different as shown in Figure 17. The most abundant anthocyanidin was malvidin in Cabernet Franc grape berries (335.30 mg/kg) and Pandol black seedless table grape berries (215.36 mg/kg). In Noiret grape berries the most abundant anthocyanidin was petunidin (861.08 mg/kg). In contrast, cyanidin was the dominant anthocyandin (18.48 mg/kg) in Pandol

Red Seedless Table Grape berries. Only trace amounts of malvidin was detected in Pandol Red Seedless Table Grape skins.

3.4.3 Recovery and Repeatability

The recovery of this method was validated by spiking known quantities of anthocyanidins standards, dephinidin, petunidin, cyanidin, malvidin and peonidin , corresponding approximately to 100%, 75% and 50% of the expected values in a representative grape juice sample 100% Welch's Concord grape juice concentrated prior to hydrolysis and then were together hydrolyzed using the same procedure developed. The recovery study was conducted as triplicates over three different days. The average recovery percentages were 98.59% for dephinidin, 103.20% for petunidin, 102.90% for cyanidin, 99.89% for malvidin and 102.84% for peonidin as shown in Table 6. No significant difference was observed among the recoveries when spiking different quantity of anthocyanidin standards. The RSD of the recovery with the same method is 5.03% for dephinidin, 3.30% for petunidin, 1.97% for cyanidin, 2.02% for malvidin, and 0.68% for peonidin. These validation studies indicated that the newly developed method was reliable, precise and sensitive for the quantitation of five major anthocyanidins in the grapes or grape juices samples.

Table 7. Recoveries of dephinidin, petunidin, cyanidin, malvidin and peonidin at different added level

Analyte	Concentration (umol/l)	Added (μmol/L)	Measured (μmol/L)	Recovery (%)	Average Recovery (%)	RSD (%)
Dephinidin	33.45	29.23	62.98 ± 1.10	100.48	98.59	5.03
		22.32	57.07 ± 0.75	102.33		
		12.22	42.46 ± 0.82	92.97		
Petunidin	42.56	50.31	93.67 ± 0.84	100.85	103.20	3.30
		38.67	87.01 ±0.19	107.11		
		25.36	69.04 ± 1.74	101.64		
Cyanidin	55.44	50.36	106.62 ± 0.65	100.78	102.90	1.97
		37.96	97.90 ± 0.62	104.81		
		25.57	83.52 ± 0.20	103.11		
Malvidin	20.17	27.27	46.45 ± 0.11	97.93	99.89	2.02
		20.00	40.95 ± 0.21	101.95		
		13.33	33.43 ± 0.53	99.79		
Peonidin	9.97	11.88	22.34 ± 0.38	102.23	102.84	0.68
		8.32	18.94 ± 0.71	103.60		
		5.94	16.34 ± 0.07	102.70		

3.5 CONCLUSIONS

A simple, precise and reliable acid assisted hydrolysis method was established for the quantitation of anthocyanidins in grape juice samples, grape berries and grape skins using LC/MS. Under optimized conditions, five anthocyanidins including delphinidin, cyanidin, petunidin, peonidin and malvidin were fully separated within 25 min and successfully quantitated. The validation of this method showed that the recovery percentages of five anthocyanidins ranged from 98.59 % to 103.20% with the relative standard deviation (RSD) less than 5.03%. With the method developed, 15 grape juices and 4 grape berries and grape skins were investigated to assess the anthocyanidins compositions across the different grape juice samples, grape berries and grape skins. These results also revealed that the total concentrations of anthocyanidins were not symmetrically distributed in the various brands of grape. Rather, among all fifteen grape juices, peonidin was found to be present in lowest concentration. The quantitative distribution of anthocyanidins in grape berries and skins were quite similar, although grape skins' concentration of anthocyanidins was four to eight times higher than their corresponding berries. This method provides a rapid and accurate tool to quantitatively study individual anthocyanidins in grapes or grape juices, furthermore to facilitate the evaluation and comparison of new commercial grapes or grape juice pruducts in market.

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CHAPTER 4

CONCLUSIONS

A rapid and comprehensive qualitative method was developed to identify and characterize the different classes of polyphenols, including anthocyanins, flavonols, phenolic acids and flavanols/proanthocyanidins, in grapes, grape juices, wines and grape-produced dietary supplements. The detection was achieved by two runs with same LC gradient but different MS ionization mode and mobile phase modifiers. Anthocyanins and flavonols were detected under positive mode with 0.4% TFA (v/v) as mobile phase modifier in water and in acetonitrile. Phenol acids and flavonols were detected under negative mode with 0.1% FA (v/v) as mobile phase modifier in water and in acetonitrile. A total of 53 compounds were identified, including 33 anthocyanins, 12 flavonols, 4 phenolic acids and 4 flavanols/proanthocyanidins. With this method developed, 15 grape juice products, 4 grape berries and skins, 3 wines and 3 grape-produced dietary supplements were qualitatively investigated to assess and compare the profiles of polyphenols among them. This new method provides a rather comprehensive profile of different class of polyphenols in grapes and other grape-derived products. This approach is rapid and can be used for the control of new grape-related products' quality, and the evaluation of parameters affecting the polyphenol compositions and accumulations during production, harvest, processing and storage.

In this research, a simple, precise and reliable acid assisted hydrolysis method was also established for the quantitation of anthocyanidins in grape juice samples, grape berries

and grape skins. Under optimized conditions, five anthocyanidins, including delphinidin, cyanidin, petunidin, peonidin and malvidin, were eluted successfully separated within 25 min. The validation of this method showed that the recovery percentages of five anthocyanidins ranged from 98.59 % to 103.20% with the relative standard deviation (RSD) less than 5.03%. Applying this newly developed quantitative method, 15 grape juice products and four grape berries and skins were chemically analyzed to assess the anthocyanidins compositions. Results showed that the method worked, and that the total concentrations of anthocyanidins were not symmetrically distributed in the various brands of grape. Peonidin was found in the lowest concentration among five anthocyanidins. The quantitative distribution of anthocyanidins in grape berries and skins were quite similar, although grape skins' concentration of anthocyanidins was four to eight times higher than their corresponding berries. This method provides a rapid and accurate tool to quantitatively study individual anthocyanidins in grapes or grape juices, furthermore to facilitate the evaluation and comparison of new commercial grapes or grape juices products in the marketplace.

APPENDIX I

ADITIONAL FIGURES IN SUPPORT OF CHAPTER 2

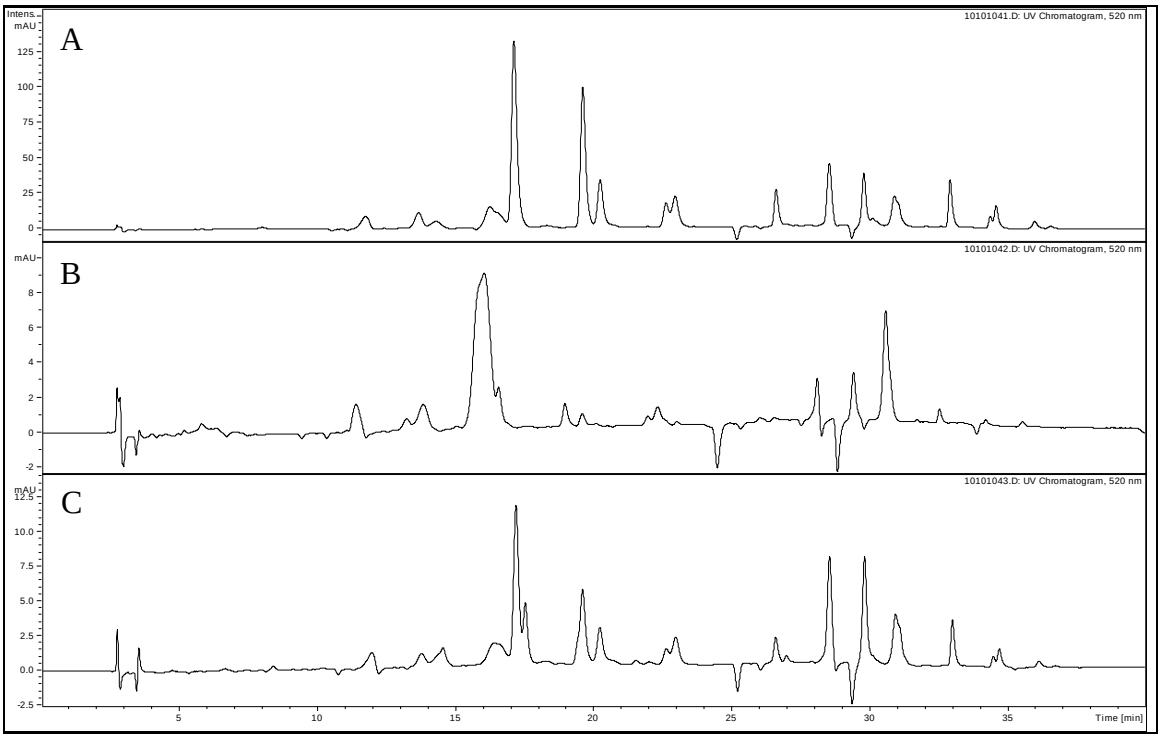

Figure 18. UV chromatograms (520 nm) of

(A) Welch's Concord Grape Juice 100% concentrated, (B) Welch's Concord Grape Juice Cocktail, and (C) Welch's Light Concord Grape Juice.

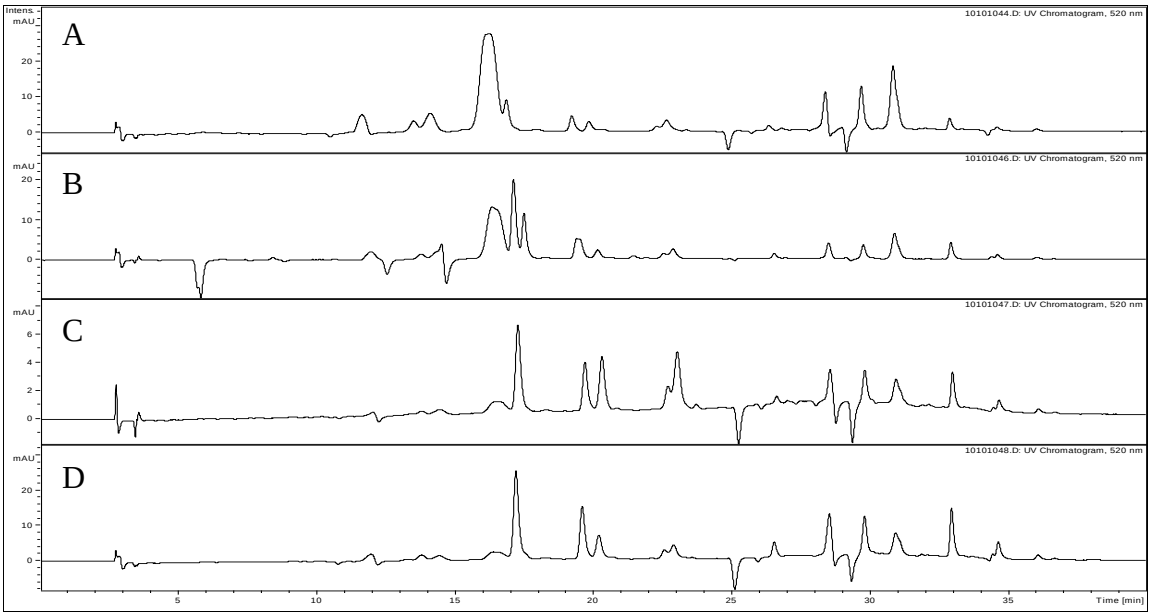

Figure 19. UV chromatograms (520 nm) of (A) Welch's 100% Black Cherry Concord Grape Juice, (B) Langers Pomegranate Grape juice, (C) Healthy Balance Grape Juice, (D) Wild Harvest Organic Grape Juice.

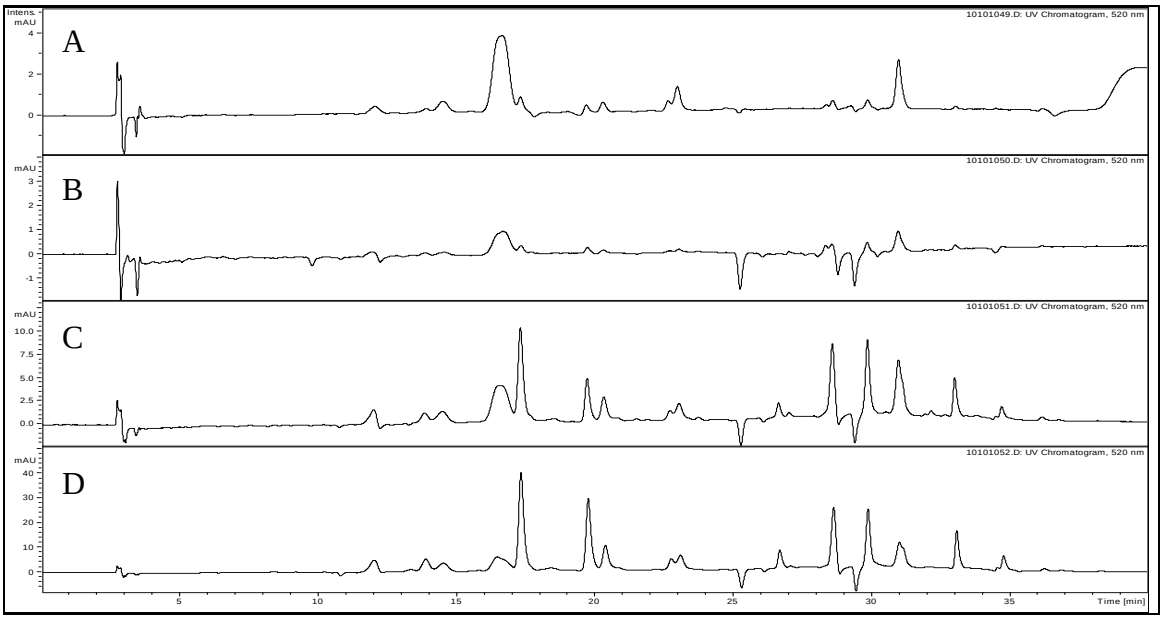

Figure 20. UV chromatograms (520 nm) of (A) Shoppers Value Grape Drink, (B) ACME Grape Juice Cocktail, (C) ShopRite Pasteurized Grape Juice, and (D) Santa Cruz Organic Concord Grape Juice.

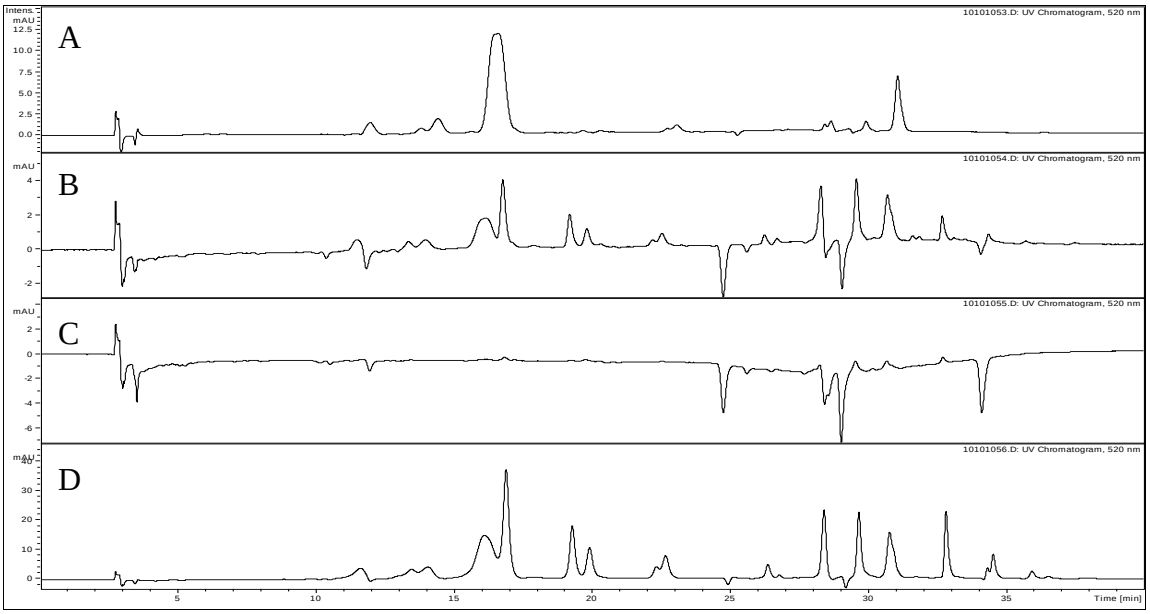

Figure 21. UV chromatograms (520 nm) of (A) Snapple Naturally Flavored Grapeade Juice Drink, (B) Walgreens Grape Juice, (C) Manischewitz Premium Grape Juice, (D) Kedem Concord Grape Juice.

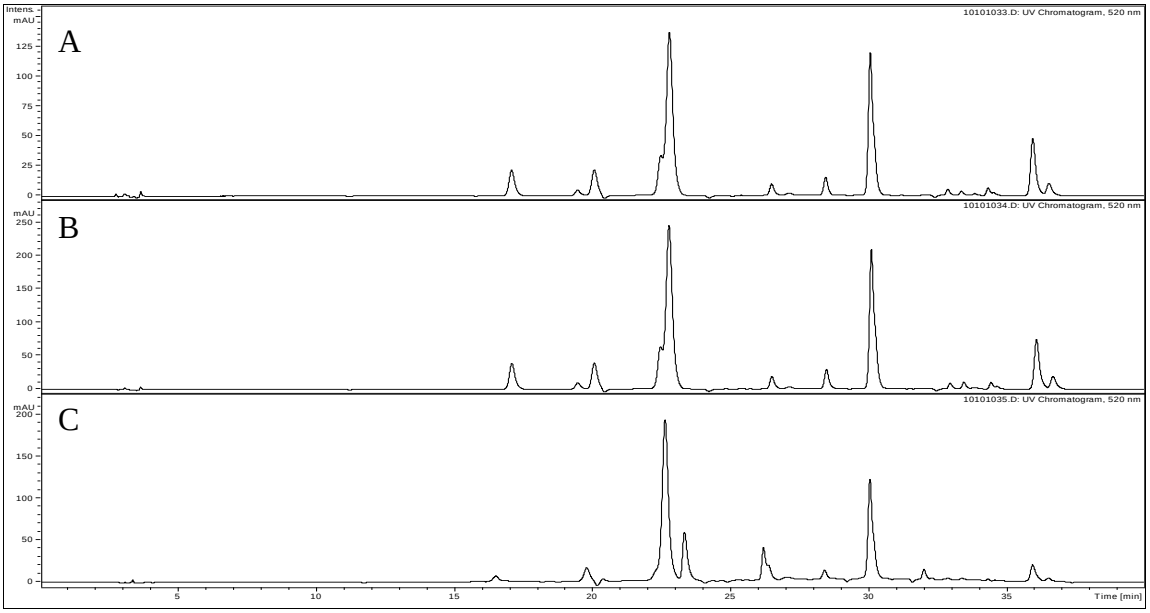

Figure 22. UV chromatograms (520 nm) of (A) Cabernet Franc Grape Berries, (B) Cabernet Franc Grape Skins and (C) Cabernet Franc Wine.

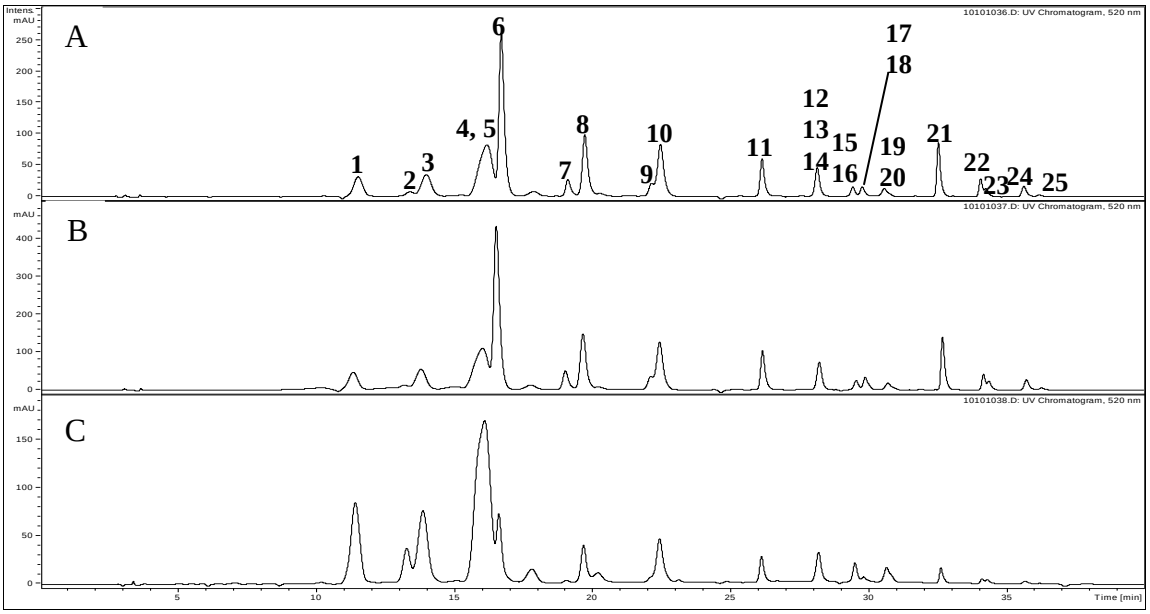

Figure 23. UV chromatograms (520 nm) of (A) Noiret Grape Berries, (B) Noiret Grapes Skins, and (C) Noriet Wine.

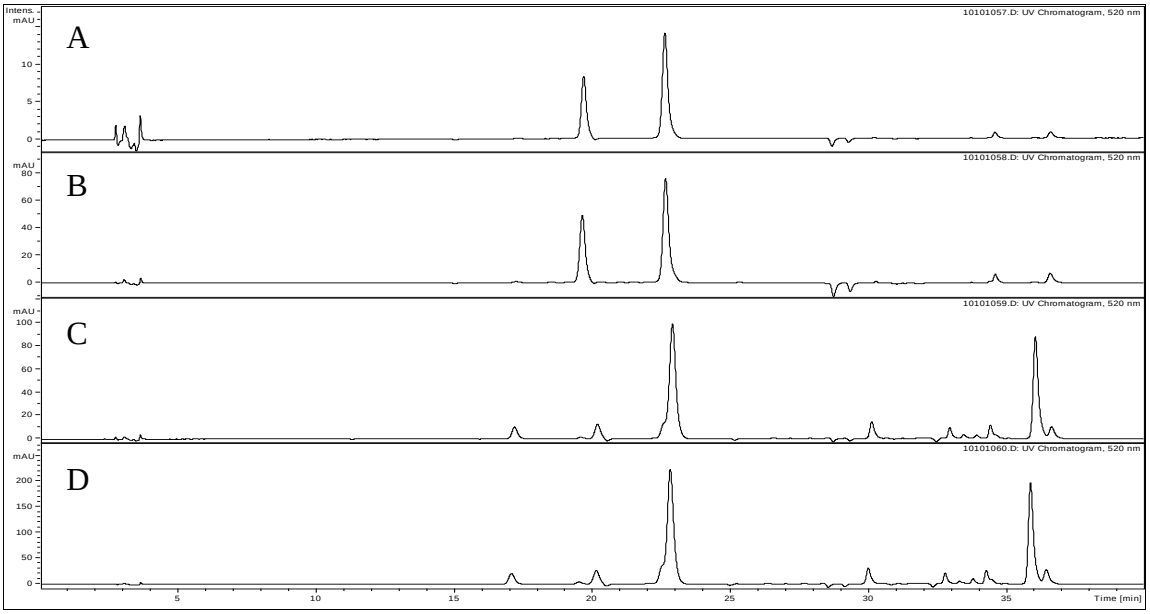

Figure 24. UV chromatograms (520 nm) of (A) Pandol Red Seedless Grape Berries, (B) Pandol Red Aeedless Grape Skins, (C) Pandol Black Seedless Grape Berries, and (D) Pandol Black Seedless Grape Skins.

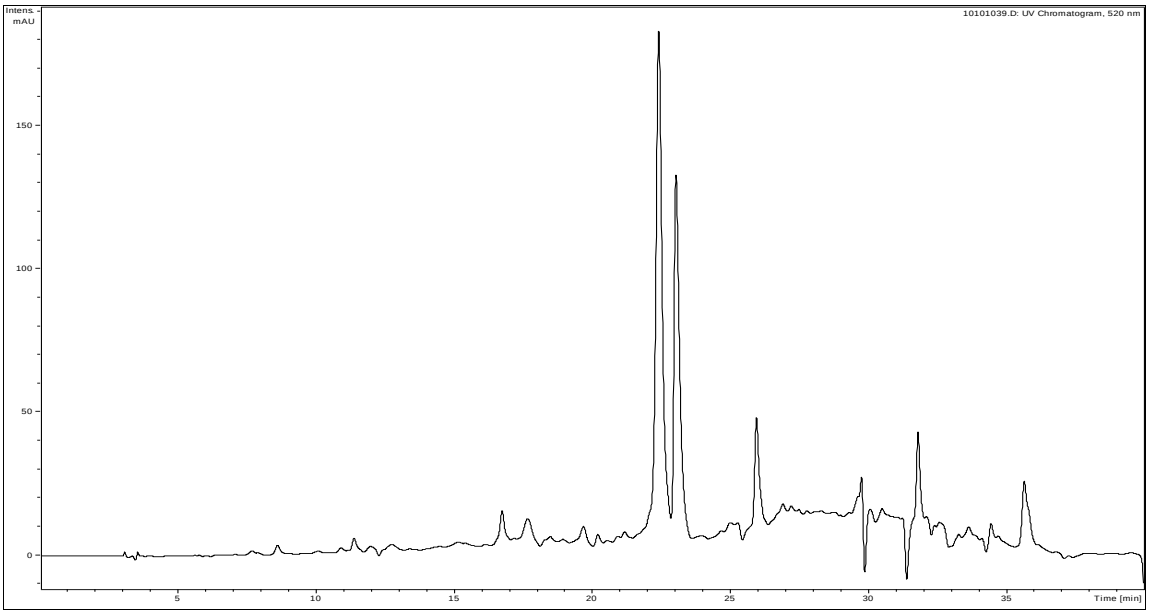

Figure 25. UV chromatogram (520 nm) of Cabernet Sauvignon Wine

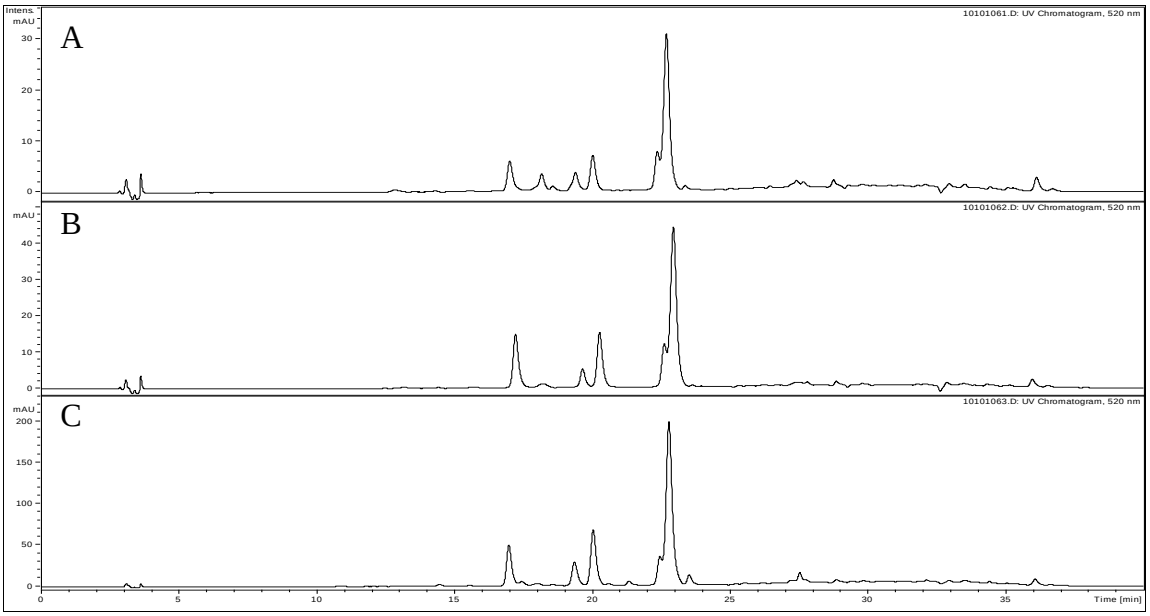

Figure 26. UV chromatograms (520 nm) of (A) Grape Complete With Pine Bark,

(B) Best French Red Wine and (C) Herbal Actives Red Wine.

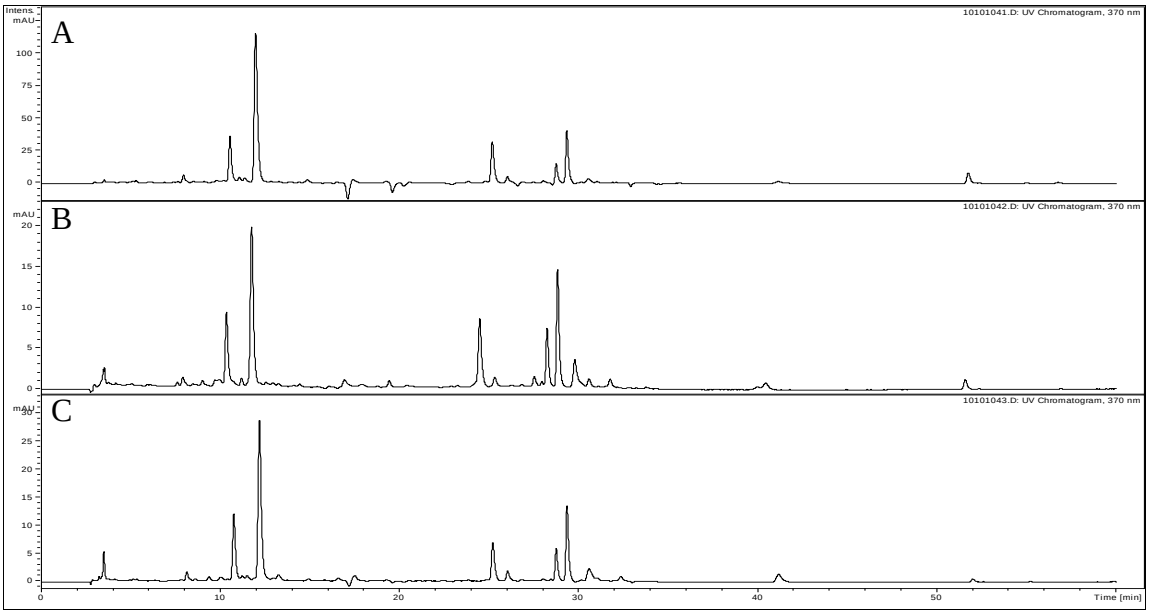

Figure 27. UV chromatograms (370 nm) of (A) Welch's Concord Grape Juice100% concentrated, (B) Welch's Concord Grape Juice Cocktail, and (C) Welch's Light Concord Grape Juice Beverage.

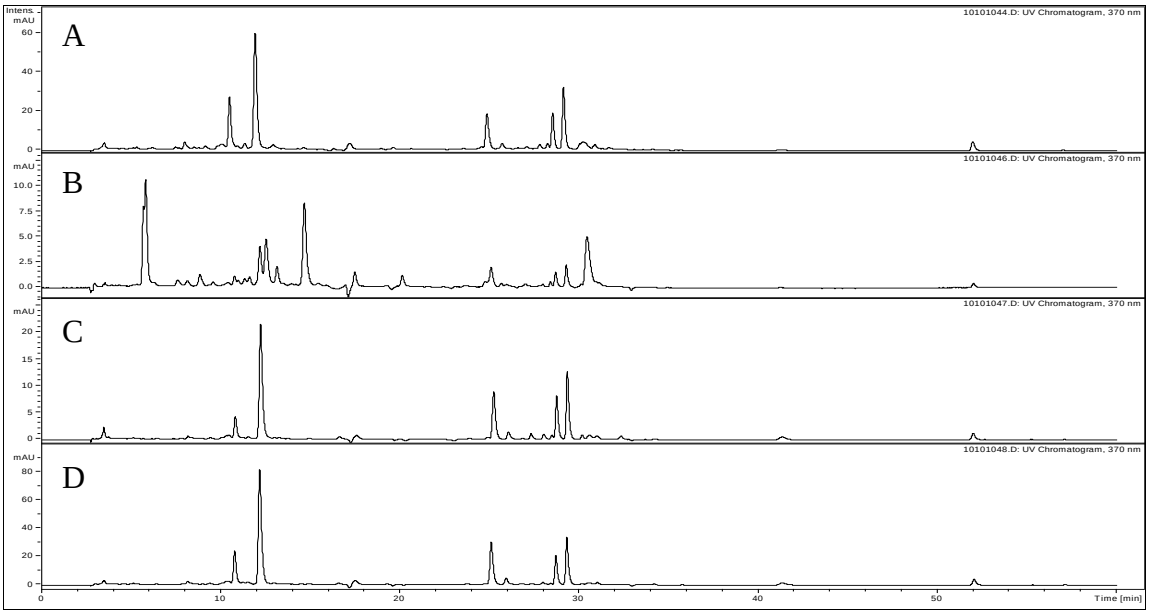

Figure 28. UV chromatograms (370 nm) of (A) Welch's 100% Black Cherry Concord Grape Juice, (B) Langers Pomegranate Grape Juice, (C) Healthy Balance Grape Juice, (D) Wild Harvest Organic Grape Juice

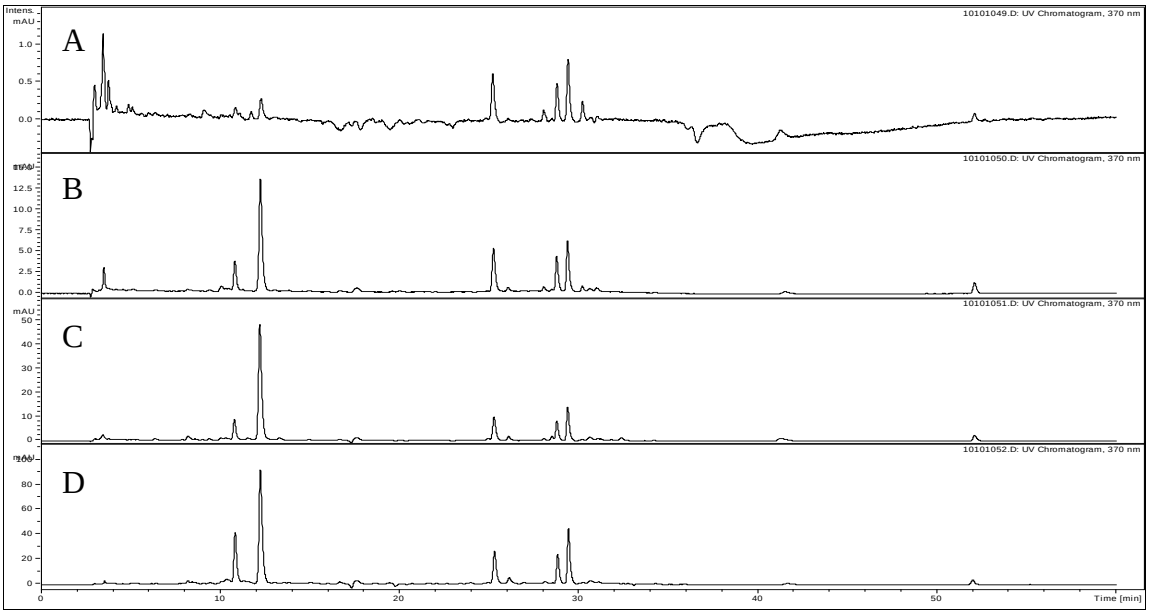

Figure 29. UV chromatograms (370 nm) of (A) Shoppers Value Grape Drink; (B) ACME Grape Juice Cocktail; (C) ShopRite Pasteurized Grape Juice; (D) Santa Cruz Organic Concord Grape Juice.

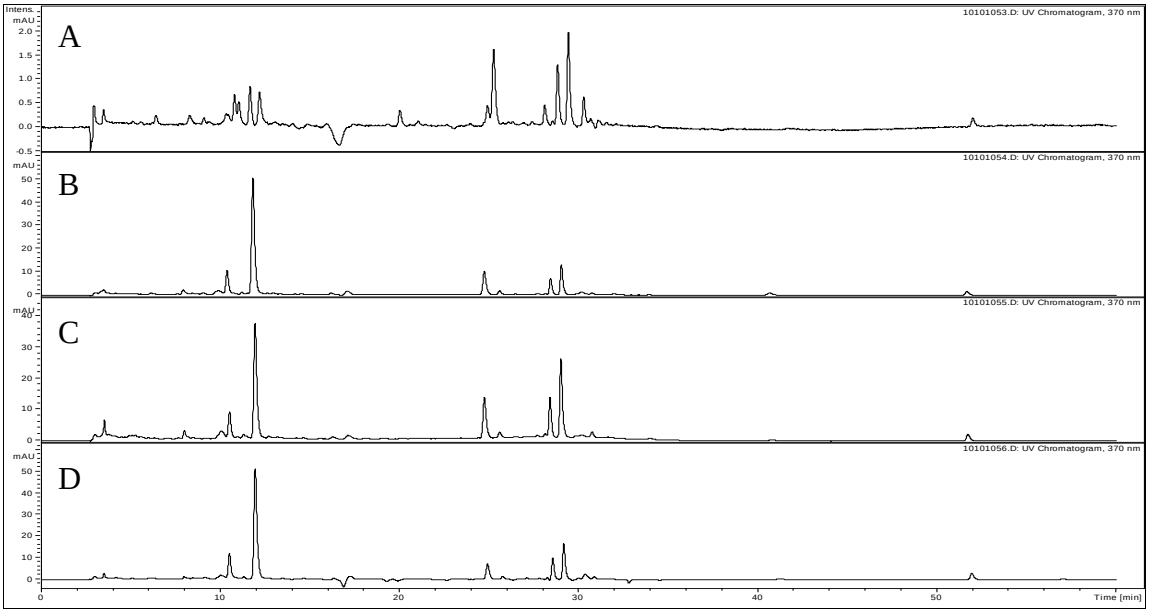

Figure 30. UV chromatograms (370 nm) of (A) Snapple Naturally Flavored Grapeade Juice Drink, (B) Walgreens Grape Juice, (C) Manischewitz Premium Grape Juice, (D) Kedem Concord Grape Juice.

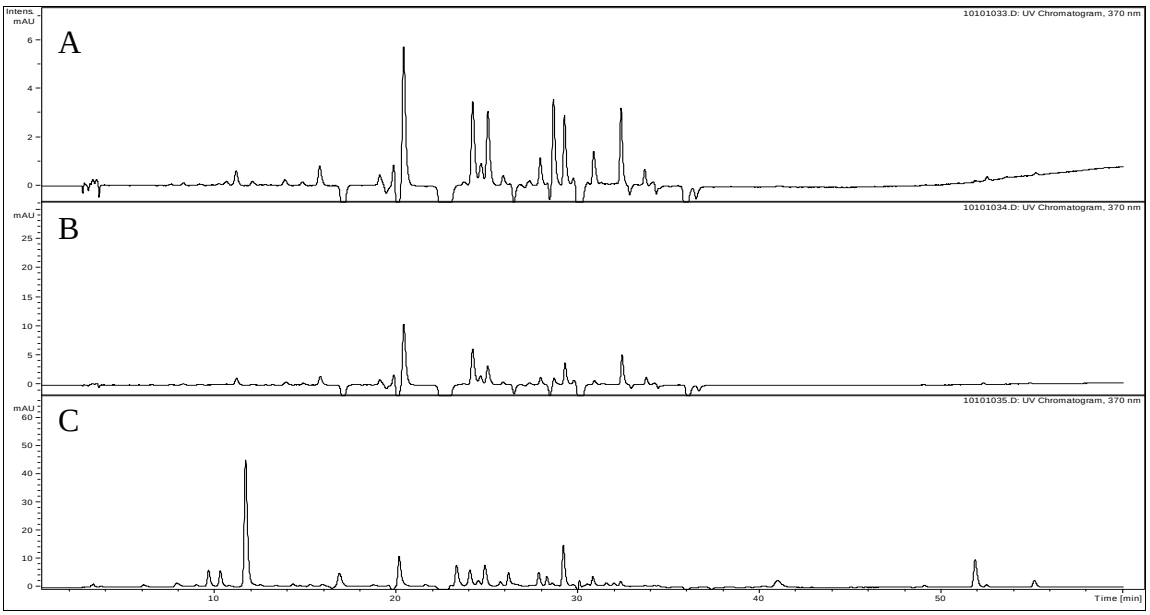

Figure 31. UV chromatograms (370 nm) of (A) Cabernet Franc Grape berries, (B) Cabernet Franc Grape Skins and (C) Cabernet Franc Wine.

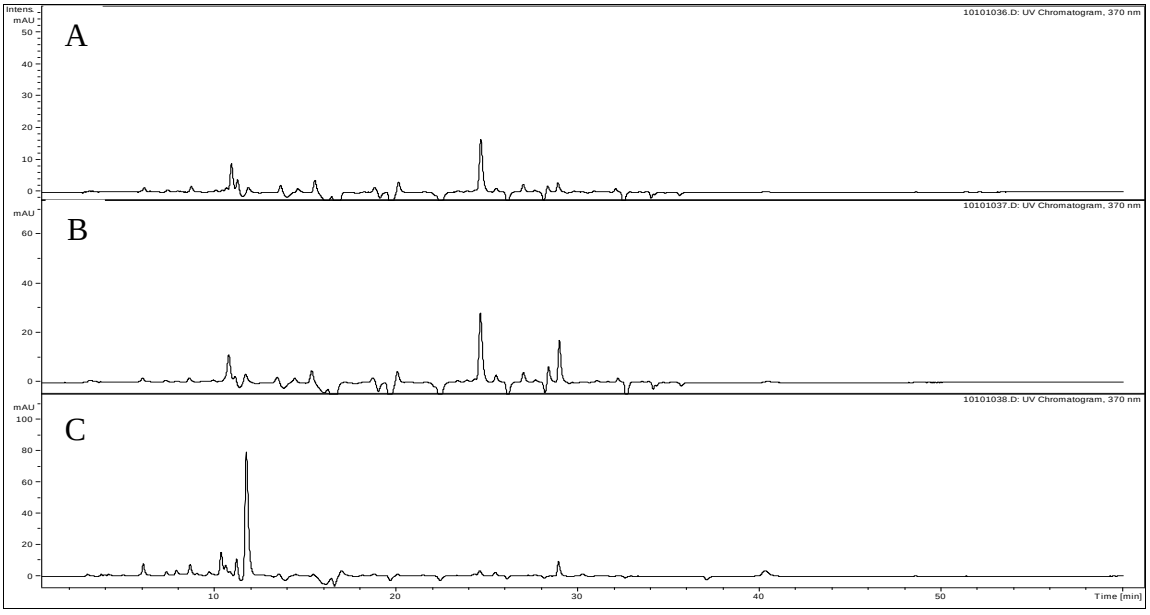

Figure 32. UV chromatograms (370 nm) of (A) Noiret Grape Berries, (B) Noiret Grapes Skins, and (C) Noriet Wine.

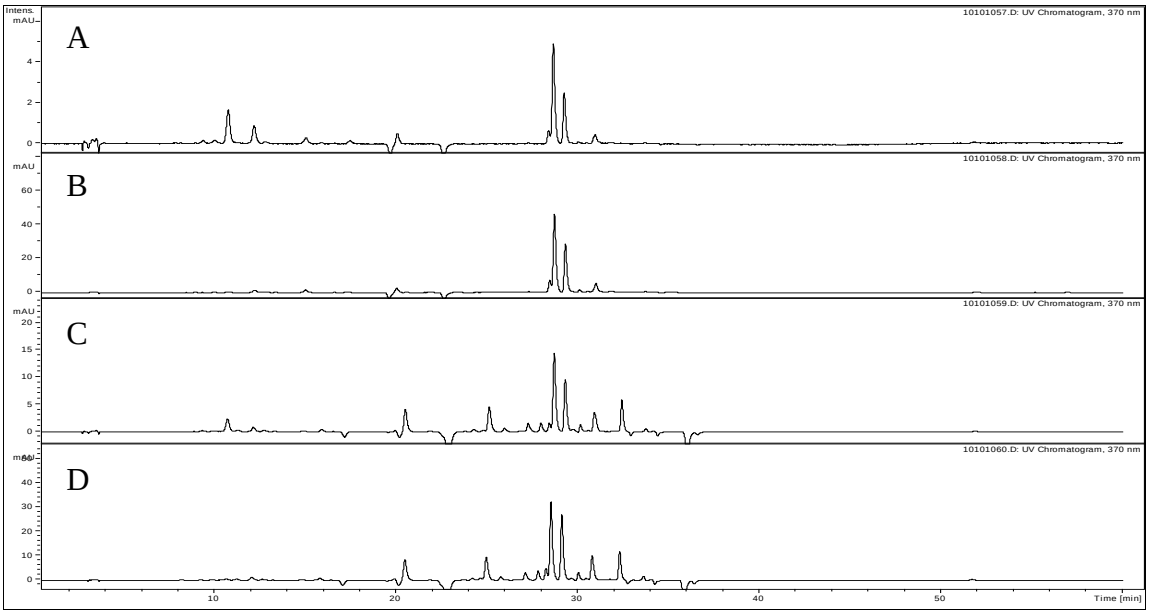

Figure 33. UV chromatograms (370 nm) of (A) Pandol Red Seedless Grape Berries, (B) Pandol Red Seedless Grape Skins, (C) Pandol Black Seedless Grape Berries, and (D) Pandol Black Seedless Grape Skins.

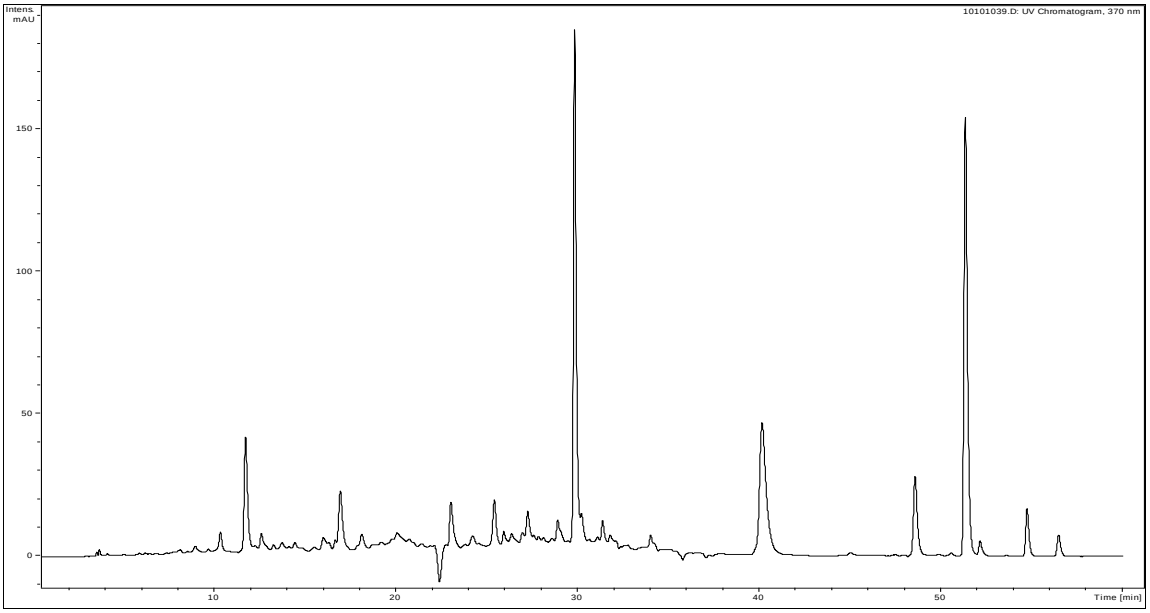

Figure 34. UV chromatogram (370 nm) of Cabernet Sauvignon Wine.

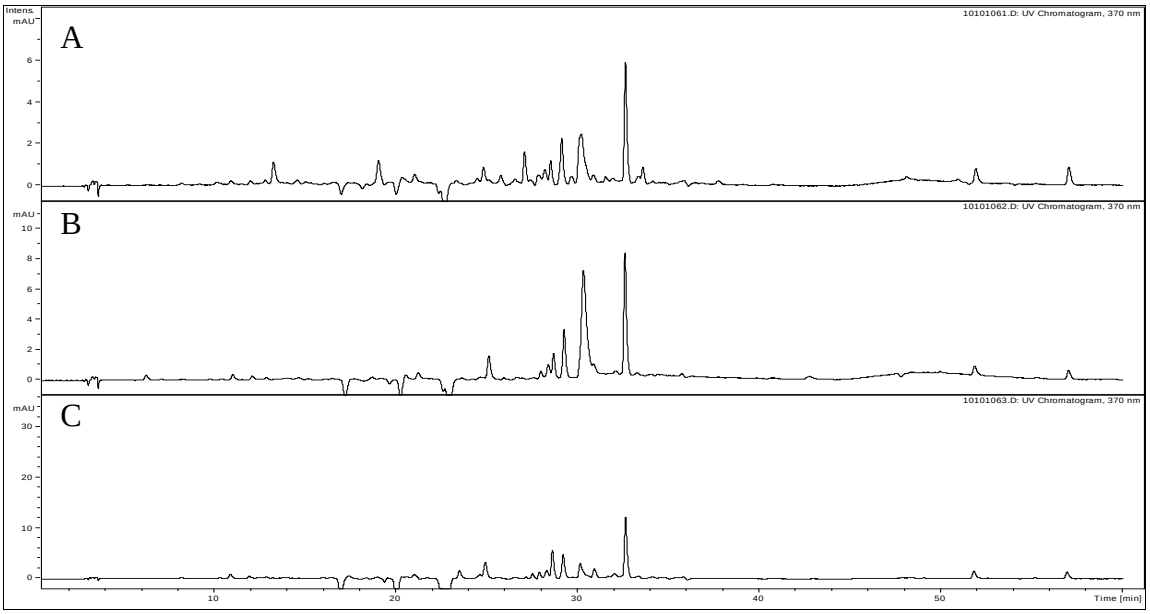

Figure 35. UV chromatograms (370 nm) of (A) Grape Complete with Pine Bark, (B) Best French Red Wine and (C) Herbal Actives Red Wine.

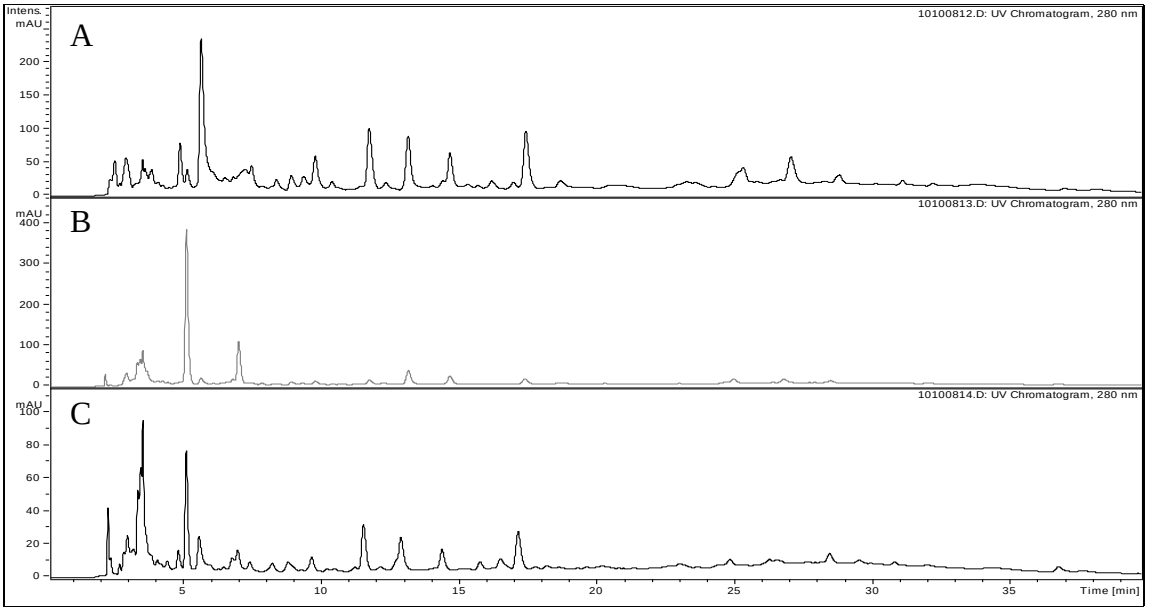

Figure 36. UV chromatograms (280 nm) of (A) Welch's Concord Grape Juice 100% concentrated, (B) Welch's Concord Grape Juice Cocktail, and (C) Welch's Light Concord Grape Juice Beverage.

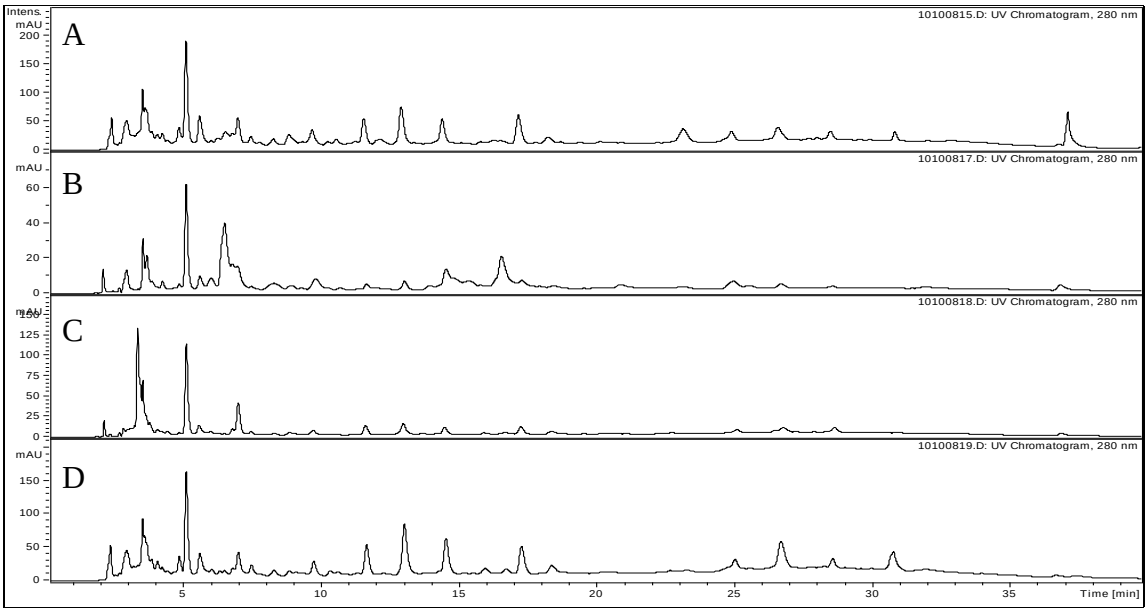

Figure 37. UV chromatograms (280 nm) of (A) Welch's 100% Black Cherry Concord Grape Juice, (B) Langers Pomegranate Grape Juice, (C) Healthy Balance Grape Juice, and (D) Wild Harvest Organic Grape Juice.

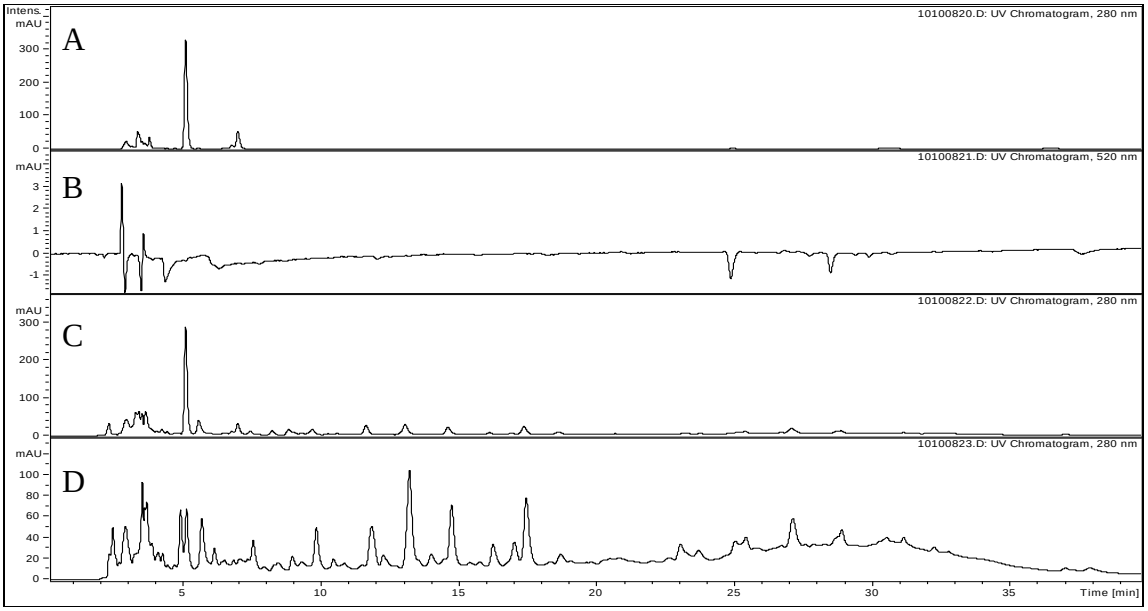

Figure 38. UV chromatograms (280 nm) of (A) Shoppers Value Grape Drink, (B) ACME Grape Juice Cocktail, (C) ShopRite Pasteurized Grape Juice, and (D) Santa Cruz Organic Concord Grape Juice.

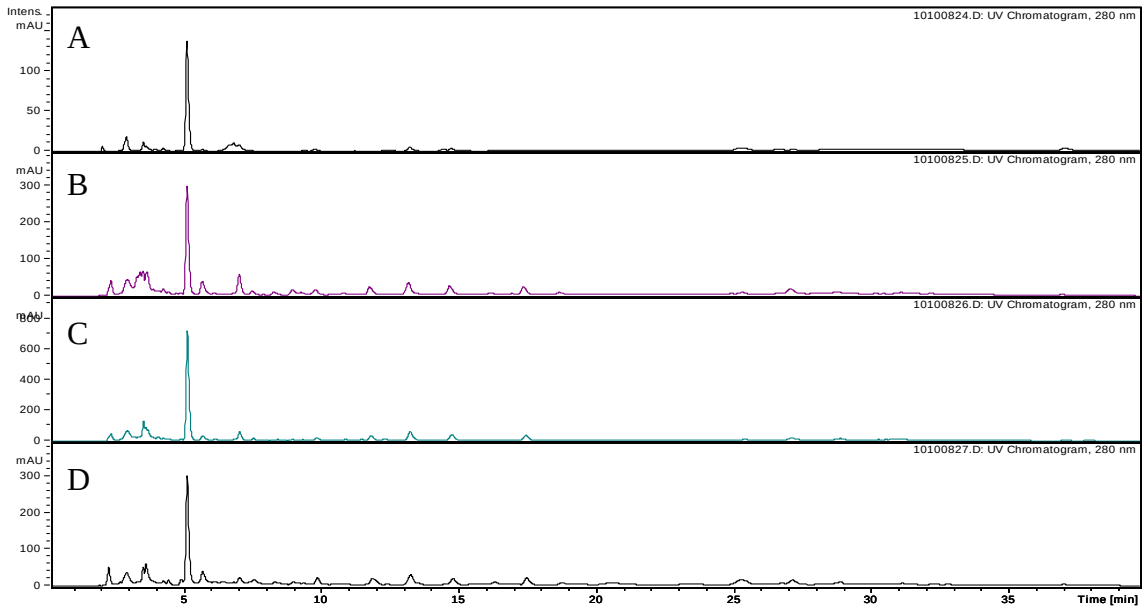

Figure 39. UV chromatograms (280 nm) of (A) Snapple Naturally Flavored Grapeade Juice Drink, (B) Walgreens Grape Juice, (C) Manischewitz Premium Grape Juice, and (D) Kedem Concord Grape Juice.

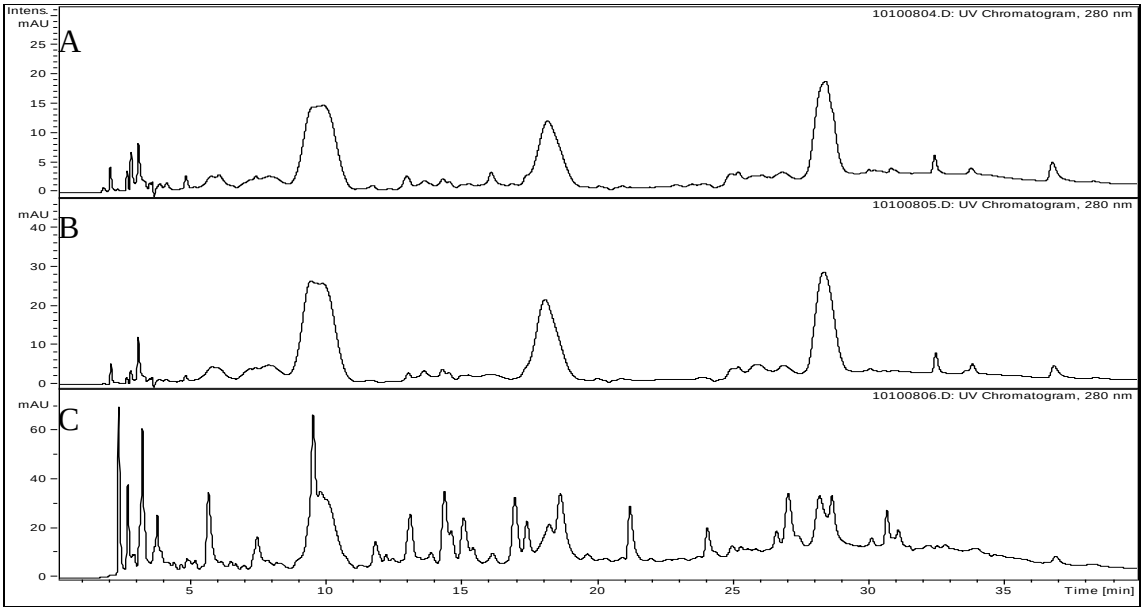

Figure 40. UV chromatograms (280 nm) of (A) Cabernet Franc Grape Berries, (B) Cabernet Franc Grape Skins and (C) Cabernet Franc Wine.

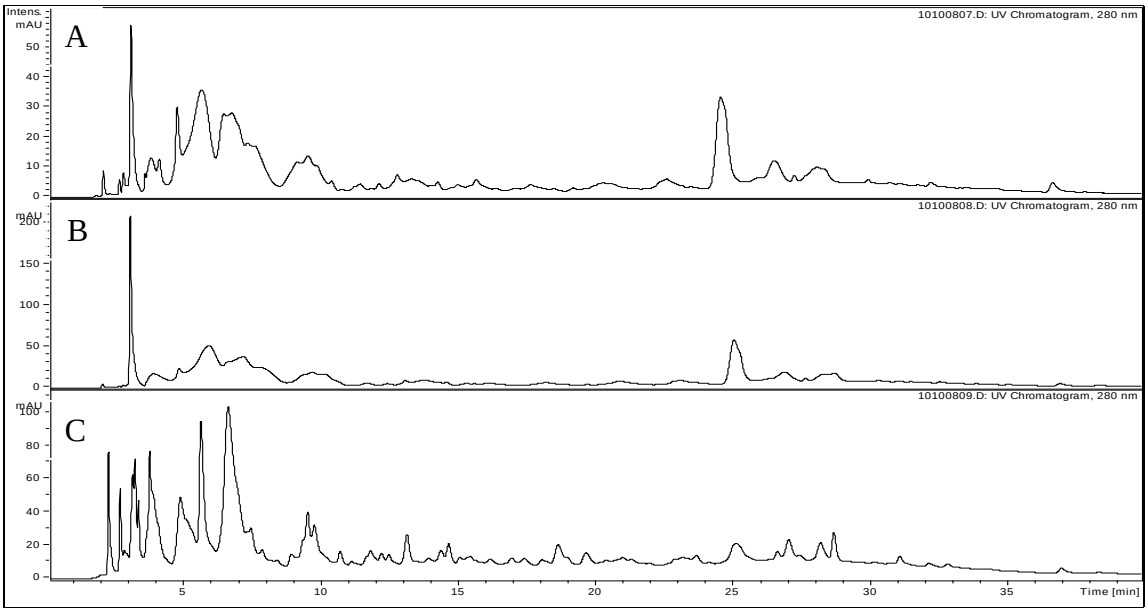

Figure 41. UV chromatograms (280 nm) of (A) Noiret Grape Berries, (B) Noiret Grapes Skins, and (C) Noriet Wine.

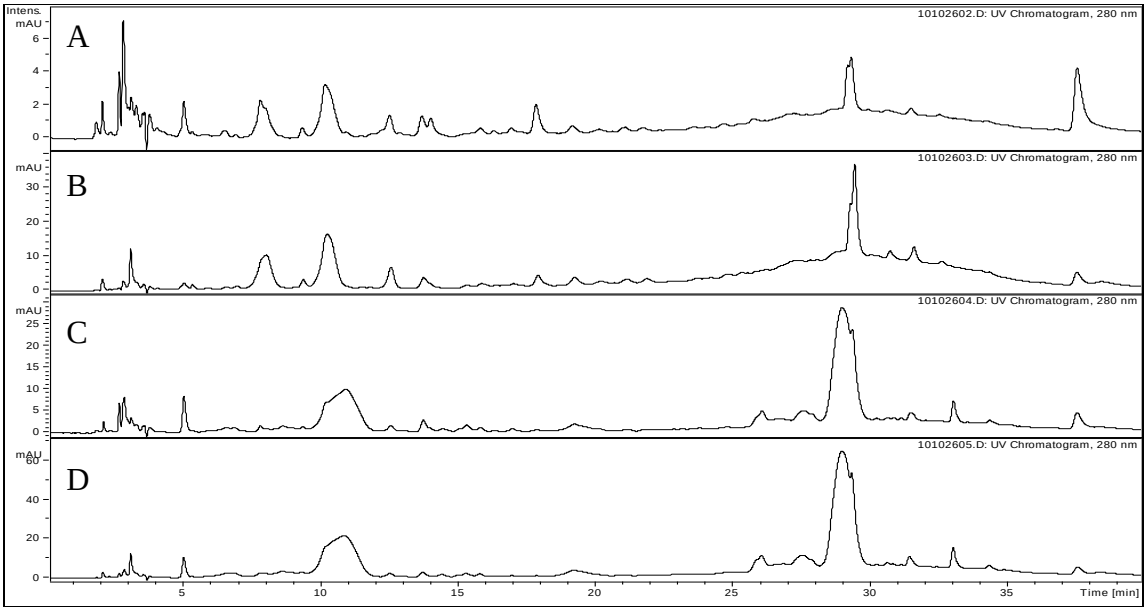

Figure 42. UV chromatograms (280 nm) of (A) Pandol Red Seedless Grape Berries, (B) Pandol Red Seedless Grape Skins, (C) Pandol Black Seedless Grape Berries, and (D) Pandol Black Seedless Grape Skins.

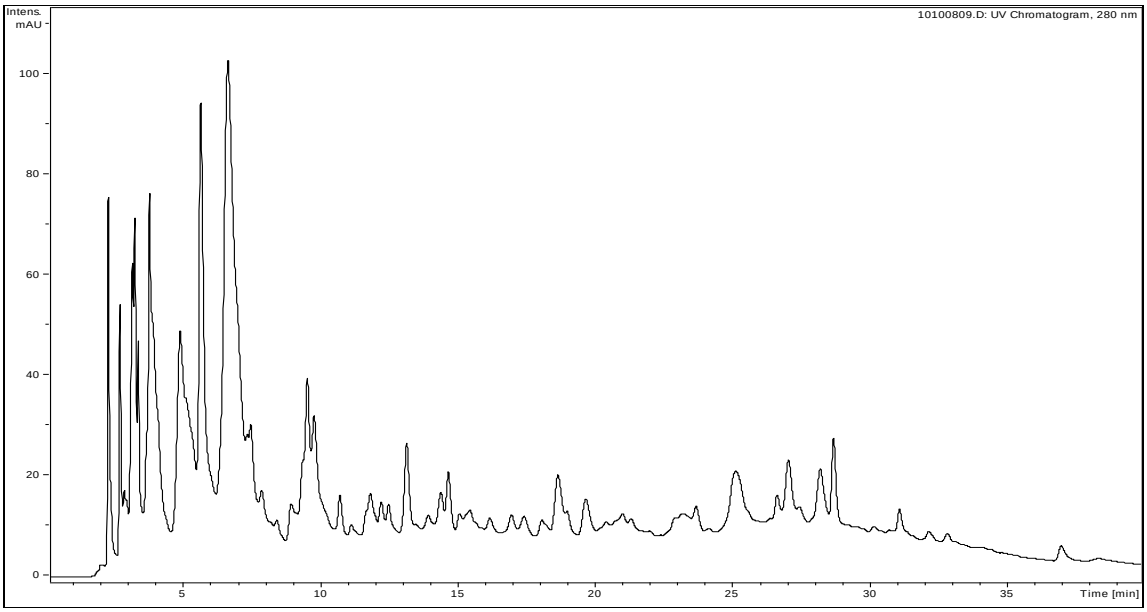

Figure 43. UV chromatogram (280 nm) of Cabernet Sauvignon Wine.

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APPENDIX II

PHYTOCHEMICAL STUDY AND BIOACTIVE PROPERTIES OF EYEBRIGHT (*Euphrasia officinalis*)

1 ABSTRACT

Eyebright (*Euphrasia officinalis*) has a long history of herbal use for the treatment of conjunctivitis, blepharitis and inflammation of the upper respiratory passages, hay fever, colds in Europe. High-performance liquid chromatography coupled with ultraviolet and electrospray ionization mass spectrometer (HPLC/UV/MS) was used to characterize the chemical constituents in the aerial part. Under optimized conditions, the aqueous methanol extract of eyebright was chemically profiled and a total of more than 28 compounds were identified by interpretation of their UV and MS data, and also by comparison with the standards. In this investigation, different bioactive parts of iridoid glycosides, phenolic acids and flavonoids were successfully fractionated from crude eyebright extract using chromatography on a polyamide column, and were tested using two antioxidant assays (TEAC and ORAC) and their inhibitory effects on nitric oxide production in the lipopolysaccharide (LPS) stimulated macrophage cell line (Raw 264.7 cells). Results showed that the highest antioxidant (ROS) activity was observed for the fraction of phenolic acids, followed by flavonoids, the eyebright crude extracts and iridoid glycosides. Flavonoids showed the highest anti-inflammatory activity. The iridoid glycosides fraction was further fractionated from which we were able to purify and identify five iridoids glycosides: strictoloside, gardoside methyl ester, ipolamiide, 7-epi-loganin and mussaenoside using MS, ^{1}H NMR and ^{13}C NMR.

2 INTRODUCTION

Eyebright (*Euphrasia officinalis*), an annual semiparasitic herb found in meadows and pastures reaches about 0.3 m in height. The plant has small, deeply toothed leaves and white or pink flowers with purple streaks and a yellow spot on the petal. [1] Native to Europe and parts of western Asia, Eyebright is one of the primary herbal sources of eye care. [2] This plant has been used for over 2,000 years in traditional medicine in the treatment of a variety of eye problems in Europe.[3] Eyebright has also been traditionally used as an astringent and has and continues to be used in the treatment of conjunctivitis, blepharitis and inflammation of the upper respiratory passages, hay fever, colds and more. [2] Despite eyebright's long history for treating eye ailments, its effectiveness has not been systematically examined and the constituents responsible for its myriad of purported bioactivities are poorly understood. [4, 5] The objective of this investigation is to chemically profile the natural products of eyebright, develop methods for separation of the different bioactive fractions and major compounds in eyebright and to evaluate the isolated fractions with respect to antioxidant activity and anti-inflammatory activity.

3 MATERIALS AND METHODS

3.1 Materials

HPLC grade acetonitrile (ACN) and methanol (MeOH) were obtained from Fisher Scientific Co. (Fair Lawn, NJ). HPLC grade formic acid was obtained from Acros Organics (NJ). HPLC-grade water was prepared using a Millipore Milli-Q purification system (Millipore Corporation, Bedford, MA). Polyamide was purchased from Sigma-

Aldrich Inc. Eyebright (*Euphrasia officinalis*) was purchased in Samllflower Inc. (Chicago, IL) as a dry aerial part.

3.2 HPLC/MS conditions

An Agilent 1100 Series LC/MSD system equipped with quaternary pump, multiple wavelength detector, MSD trap with an electrospray ion source (ESI) was utilized for LC-UV-ESI/MS experiment. A 250 X 4.6mm i.d., 8μm, Microsorb 60 C18 column (Varian, Inc., Lake Forest, CA) was used for the HPLC separation at a flow rate of 1.0mL/min. The method of HPLC was performed with mobile phase containing solvent A and B in gradient, where A was 0.1% formic acid (v/v) in water and B was 0.1% formic acid in acetonitrile. The gradient profile was: 5% B in the first 10 min, varied linearly from 5% B to 20% B in the next 10 min, held isocratic at 20% B from 20min to 40min, and finally went linearly from 20% B to 40% B for 60 min. The injection volume was 20 μL. The electrospray mass spectrometer was operated under positive ion mode and scanned from *m/z* 120 to 900. The flow rate of drying gas of pure nitrogen was 12 L/min with the 350 ℃ drying gas temperature. Nitrogen was used as nebulizer at 60 psi. Helium was used as collision gas.

3.3 Phytochemical studies

Eyebright herb (1 kg air-dried) was ground into fine particles and extracted 3X with 80% methanol (3x2000mL) at room temperature overnight. The filtrations were combined and evaporated to dryness under reduced pressure to obtain 164.9 g crude extracts. The crude extracts were then dissolved in water and partitioned 3X between chloroform and water

in a separation funnel to remove the lipophilic substances. The degree of purification was visually assessed from the coloration of the chloroform layer. The aqueous extracts were combined and evaporated to dryness under reduced pressure to obtain 162.4 g extract. The chloroform layer was combined and evaporated to dryness under reduced pressure to obtain 5.1 g extract.

The residue (162.4 g) after partition was fractionated on a polyamide column in the ascending mode with MeOH-H_2O mixture eluents (0:100, 10:90, 20:80, 40:60, 60:40, 80:20, 100:0) to obtain three fractions of total iridoid glycosides (97.2g), total phenolic acids (3g) and total flavonoids (8g). Iridoids glycosides (25g) were further separated using silica flash chromatography column with ascending gradient MeOH-$CHCl_3$ mixtures (10:90 to 100:0) yielding 15 major fractions combined with TLC and LC/MS monitoring. They were fractions C_2F_1(0.1580g), C_2F_2 (1.0351g), C_2F_3 (0.1058g), C_2F_4 (0.1001g), C_2F_5 (0.8482g), C_2F_6 (1.4477g), C_2F_7 (1.1479g), C_2F_8 (1.3994g), $C_2F_{9\text{-}23}$ (5.300g), $C_2F_{24\text{-}30}$ (2.2512g), $C_2F_{31\text{-}35}$ (0.90g), $C_2F_{36\text{-}38}$ (1.90g), $C_2F_{39\text{-}40}$ (2.01g), $C_2F_{41\text{-}42}$ (1.40g)and $C_2F_{43\text{-}47}$ (5.0g). Five fractions from them, C_2F_4 to C_2F_8, were further fractionated using preparative HPLC and led to the purification and identification of five iridoids glycosides. They are strictoloside, gardoside methyl ester, ipolamiide, 7-epi-loganin and mussaenoside and the structures were elucidated using MS, ^{1}H NMR and ^{13}C NMR. All the fractions as well as the crude eyebright extracts were subjected to chemical characterization using LC-UV-ESI/MS.

3.4 Nitrite Assay

The RAW264.7 cells were exposed extracts and LPS or LPS only. After the centrifuge, the supernatants were harvested and the amount of nitrite, an indicator of NO synthesis, was measured by use of the Griess reaction. Briefly, supernatants (100 μl) were mixed with the same volume of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) in duplicate on 96-well plates. After incubation at room temperature for 10 min, absorbance at 570 nm was measured with the ELISA reader (Thermo Labsystems Multiskan Ascent, Finland).

3.5 Antioxidant Assay

The ABTS radical reagent was prepared by adding 38.4 mg of ABTS and 6.6 mg of potassium persulfate in 10 mL of water. The ABTS radical reagent was mixed well and placed in the dark for 16-20 hrs to allow the radical to fully develop; the radical is stable in this form for more than a day when stored in the dark at room temperature. The ABTS reagent was diluted with ethanol to an absorbance of 0.70 (± 0.02) at 734 nm and equilibrated at 30 ^{o}C. 1.3 mL of ABTS reagent was added to 100 mL ethanol with more ethanol (5 mL at a time) or ABTS reagent (30 μL at a time) was added to adjust the absorbance to a range of 0.68 - 0.72. About 100 mg of extracts was extracted by sonicating in 10 mL of water for 1 hour. 10 μL of this extract and 990 μL of ABTS solution (0.71 Abs) were combined in a centrifuge tube and allowed to develop at room temperature for 20-30 min. Each sample was transferred to a cuvette and read spectrophotometrically at 734 nm. The samples were measured against a blank (1 mL of ethanol) and a reference sample, which was made by adding 10 μL ethanol to 990 μL of

ABTS solution. A calibration curve was prepared by dissolving 15.5 mg of trolox in 25 mL of pure ethanol to make the standard stock solution.

4 RESULTS AND DISCUSSIONS

4.1 Flavonoids and iridois identification by LC/MS

Aqueous MeOH (80%) extracts of eyebright samples were assayed using HPLC with UV-DAD and MS detectors scanning from *m/z* 100 to 900 under the collision energy level of 100%. A representative MS total ion chromatogram (TIC) of eyebright extract and the reconstructed MS chromatograms of flavonoids derived from five different aglycones of apigenin, luteolin, diosmetin, rhamnetin and 3, 4'-dimethoxy-5, 7, 3'-trihydroxyflavone (3-OMe-diosmetin) is illustrated in Figure 46. The structures of the labeled peak in Figure 44 were identified by analysis of UV and MS spectral data, and by comparison with the authentic standards (Table 8). The representative MS spectra of three flavonoids derived from diosmetin is illustrated in Figure 45. Peak 10 with molecular ion at *m/z* 653 and two fragment ions at *m/z* 477 ([M+H-glucuronosyl] $^{+}$) and *m/z* 301 [M+H-glucuronosyl-glucuronosyl] $^{+}$) is diosmetin diglycuronoside. Peak 19 at 33.1 min with molecular ion at *m/z* 463 and fragment ion at *m/z* 301 ([M+H-glucosyl] $^{+}$) is diosmetin glucoside. Peak 20 at 34.8 min with molecular ion at *m/z* 477 and fragment ion at *m/z* 301 ([M+H-glucuronosyl] $^{+}$) is diosmetin glucuronoside.

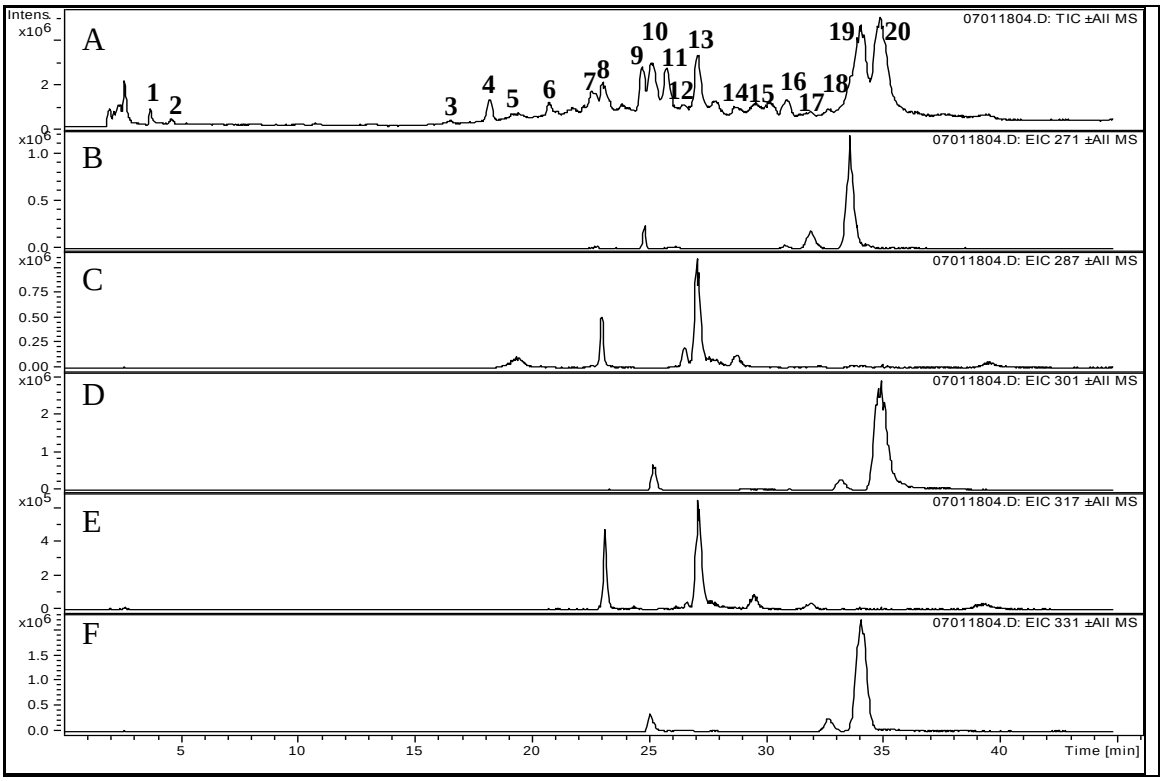


Figure 44. Representative MS total ion chromatogram of eyebright extract (A) and the extracted MS chromatograms of flavonoids derived from five different aglycones of apigenin (B, EIC: 271), luteolin (C, EIC: 287), diosmetin (D, EIC: 301), rhamnetin (E, EIC: 317) and 3-OMe-diosmetin (F, EIC: 331)

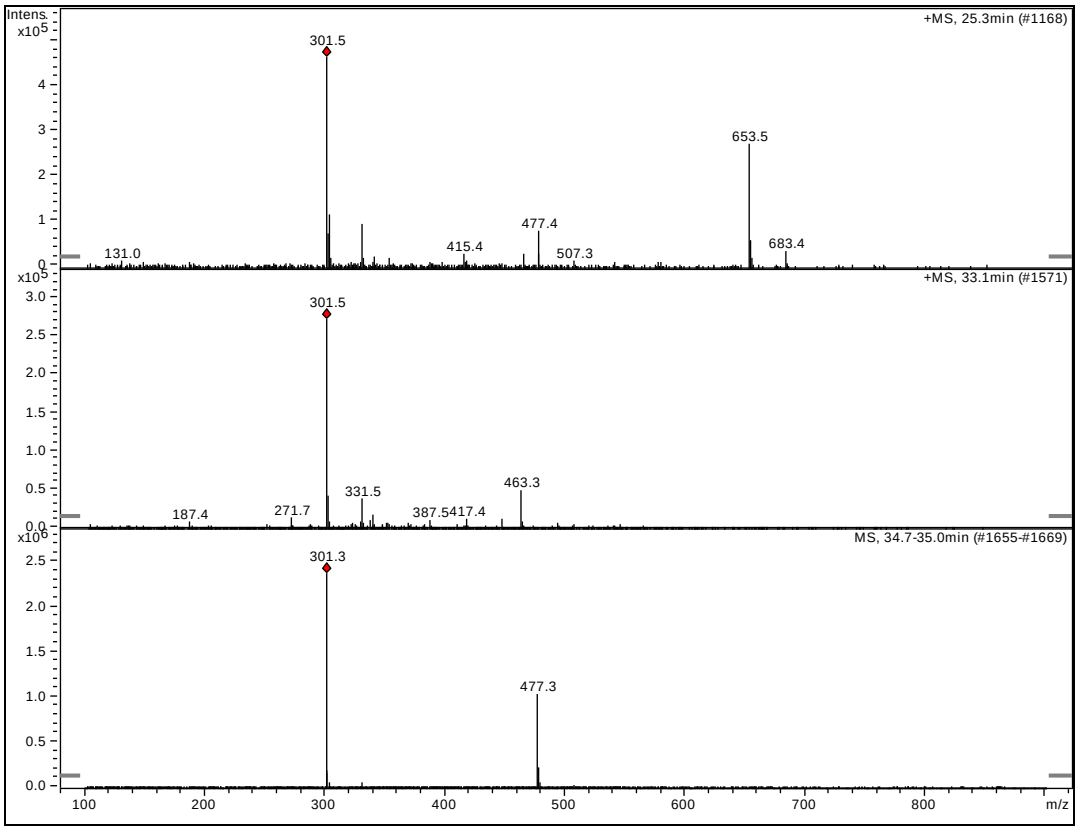
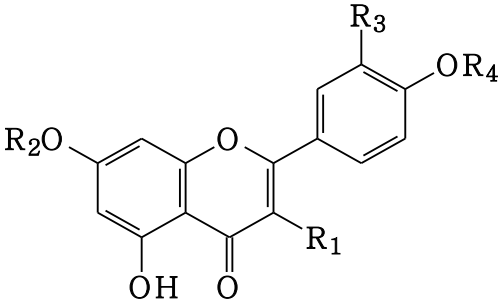


Figure 45. The specific MS spectra of three flavonoids derived from diosmetin, (A) Diosmetin-diglucuronoside, (B) Diosmetin-glucoside and (C) Diosmetin-glucuronoside



Apigenin: R_1=R_2=R_3=R_4=H
Luteolin: R_1=R_2=R_4=H, R_3=OH
Diosmetin: R_1=R_2=H, R_3=OH, R_4=Me
Rhamnetin: R_4=H, R_1=R_3=OH, R_2=Me
3-OMe-diosmetin: R_2=H, R_3=OH, R_4=Me, R_1=OMe

Figure 46. Structure of five flavonoid aglycones identified from eyebright extract

Table 8. Peak assignment for eyebright extracts

Peak	t_R (min)	$[M+H/Na]^+$ (*m/z*)	MS fragment ion (*m/z*)	Identities	Comp. Code
1*	3.7	385**		Catapol	**1**
2*	4.5	369**		Aucubin	**2**
3*	16.4	411**		Geniposide	**3**
4	18.1	429**		6-OH-adoxoside	**4**
5	19.2	595	449, 287	Luteolin-G-Rha	**5**
6	20.7	413**		Adoxoside	**6**
7	22.5	639	463, 287	Luteolin-GR-GR	**7**
7a	22.7	623	447, 271	Apigenin-GR-GR	**8**
8	22.9	639	463, 287	Luteolin-GR-GR	**9**
8a	23.1	669	493, 317	Rhamnetin-GR-GR	**10**
9	24.8	623	447, 271	Apigenin-GR-GR	**11**
10	25.0	683	507, 331	3-OMe-diosmetin-GR-GR	**12**
10a	25.2	653	477, 301	Diosmetin-GR-GR	**13**
11	25.7	471	325, 163	Skimmetin-G-Rha	**14**
12	26.4	449	287	Luteolin-G	**15**
12a	26.6	479	317	Rhamnetin-G	**16**
13	27.0	463	287	Luteolin-GR	**17**
13a	27.1	493	317	Rhamnetin-GR	**18**
14	28.7	449	287	Luteolin-G	**19**
15	29.4	479	317	Rhamnetin-G	**20**
16	30.6	473**		6-O-acetyladoxoside	**21**
16a	30.8	433	271	Apigenin-G	**22**
17	31.9	433	271	Apigenin-G	**23**
18	32.5	493	331	3-OMe-diosmetin-G	**24**
19	33.1	463	301	Diosmetin-G	**25**
19a	33.5	447	271	Apigenin-GR	**26**
19b	34.0	507	331	3-OMe-diosmetin-GR	**27**
20	34.8	477	301	Diosmetin-GR	**28**

Note: G: glucosyl, GR: glucurosyl.

4.2 Structure elucidation of iridois separated from eyebright extracts

The structures of five purified iridoid glycosides were elucidated using MS, ^{1}H NMR and ^{13}C NMR and in comparison with reported data. [6, 7] The MS of the five pure compounds, from which the molecular weight and fragmentation patterns were identified, is shown in Figure 4. Then ^{1}H NMR and ^{13}C NMR were applied and provided more information such

as total carbon numbers, chemical environments of protons and carbons in the compounds as well as the relationships between adjacent protons. Figure 1 and 2 illustrated the ^{1}H NMR and ^{13}C NMR of representative compound. Finally the combination of MS, ^{1}H NMR and ^{13}C NMR were used to confirm the structures of these five pure compounds as strictoloside, gardoside methyl ester, ipolamiide, 7-epi-loganin and mussaenoside (Figure. 47).

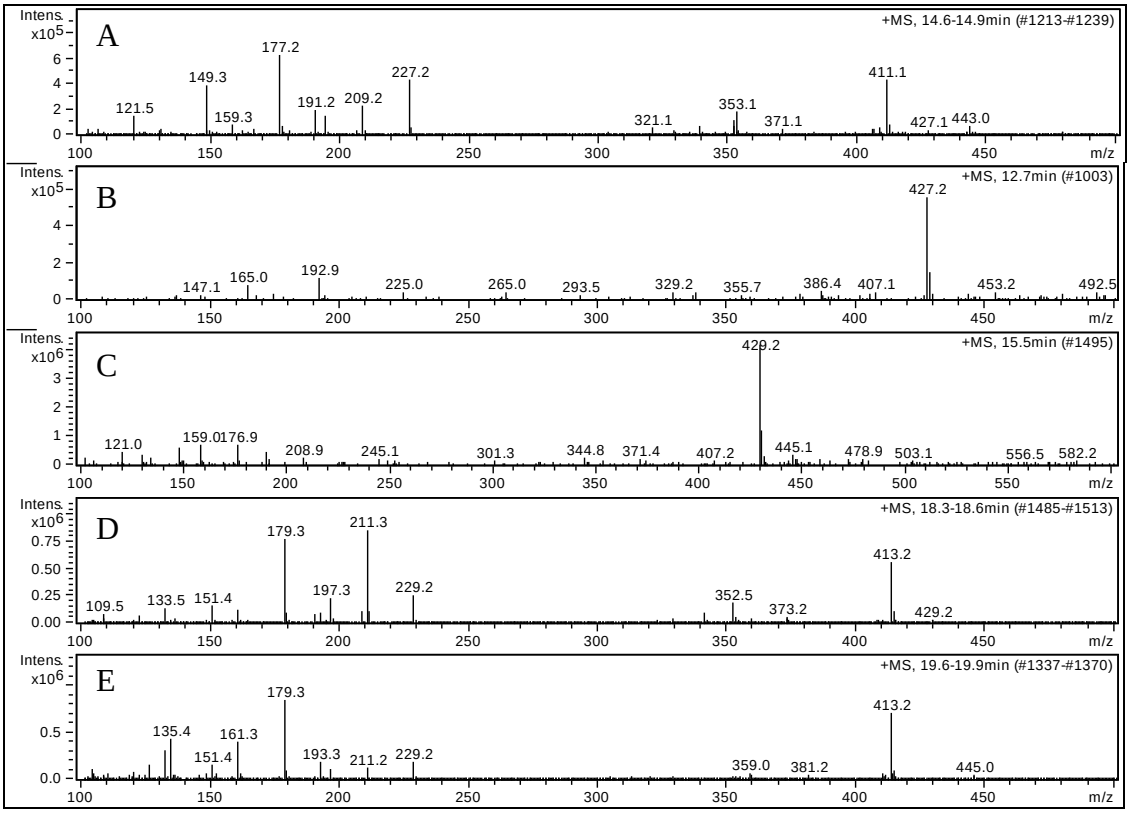


Figure 47. MS of the five purified iridoids glycosides: Strictoloside (A), Gardoside methyl ester (B), Ipolamiide (C), 7-epi-Loganin (D), and Mussaenoside (E)

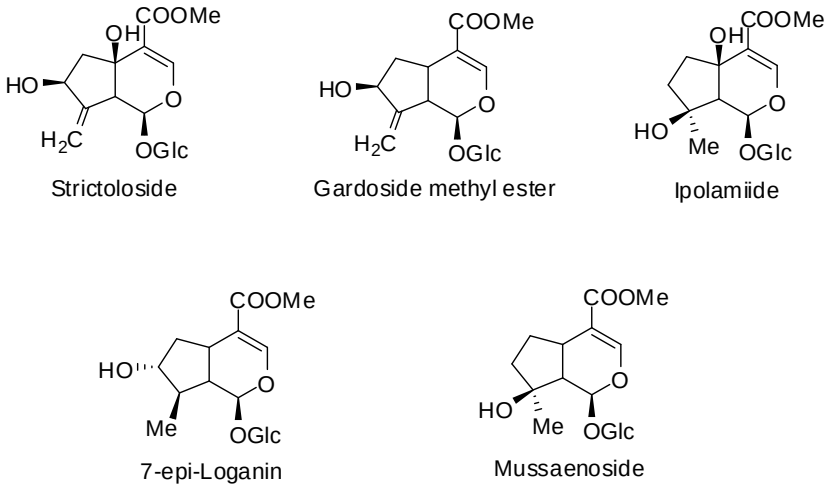


Figure 48. Structures of the identified five iridoid glycosides

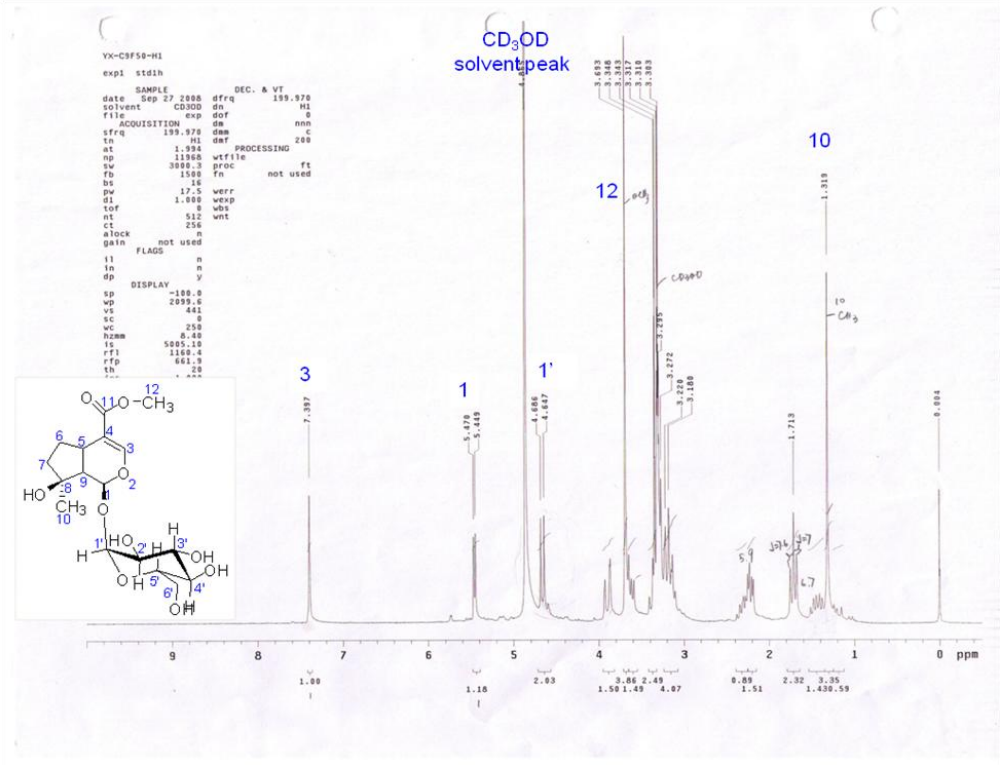


Figure 49. ^{1}H NMR of Mussaenoside

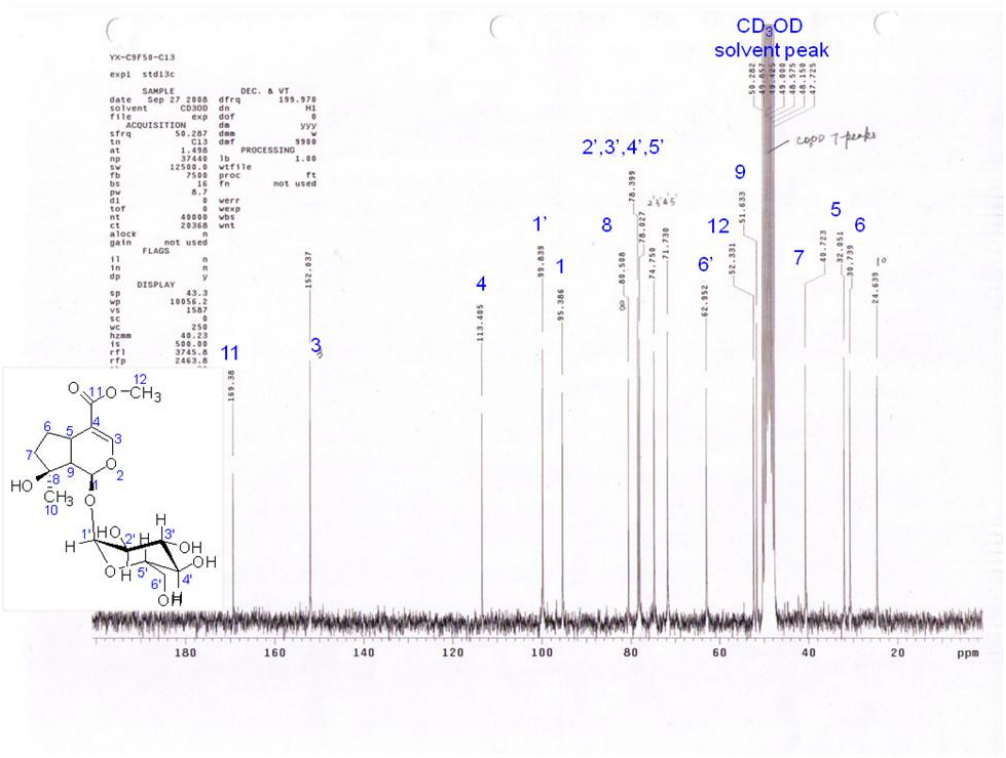


Figure 50. ^{13}C NMR of mussaenoside

4.3 Antioxidant activity investigations

Two assays were involved in the antioxidant activity investigation of eyebright crude extracts and three bioactive fractions. One was Trolox equivalent antioxidant capacity (TEAC) assay that measured the ability of antioxidants to decrease the color by prevention of the $ABTS^{+\cdot}$ radicals. The second was oxygen radical absorbance capacity (ORAC) assay that measured the prevention of the fluorescein damage from its reaction with the peroxyl radicals. Two assays with two different antioxidant (ROS) measurements generated consistent results (Figs. 51). Highest antioxidant (ROS) activity was observed for the fraction of phenolic acids, followed by flavonoids, the eyebright crude extracts and iridoid glycosides.

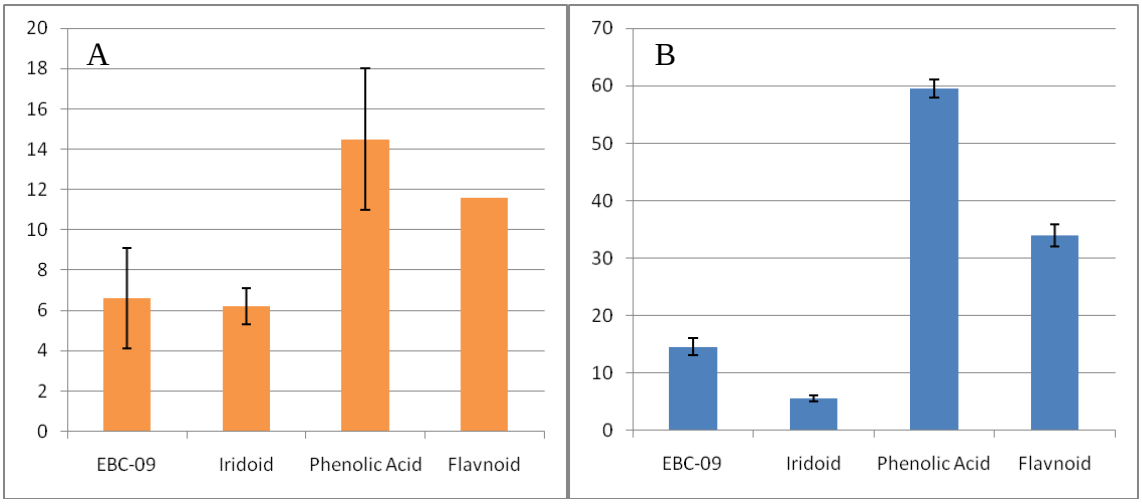


Figure 51. Antioxidant activity of eyebright extracts (crude extracts, iridoid glycoside, phenolic acids, flavonoids) as determined by (A) ORAC and (B) TEAC

Table 9. antioxidant activity (ORAC and TEAC) of eyebright extracts

Sample names	ORAC Ave. Trolox Equi. μmol/mg dry extracts	TEAC Ave.%Tro.mg /Sample mg
EBC-09	6.60 ±2.5	14.59 ±1.47
Iridoid	6.20 ±0.9	5.64 ±0.60
Phenolic Acid	14.50 ±3.5	59.52 ±1.57
Flavnoid	11.60	35.86 ±1.87

4.4 Anti-inflammatory activity investigation

Eyebright crude extracts and three fractions of iridoid glycosides, phenolic acids, and flavonoids were screened for their inhibitory effects on nitric oxide production in the lipopolysaccharide (LPS) stimulated macrophage cell line (Raw 264.7 cells). The fraction containing of total flavonoids showed the highest anti-inflammatory activity (Figure. 52).

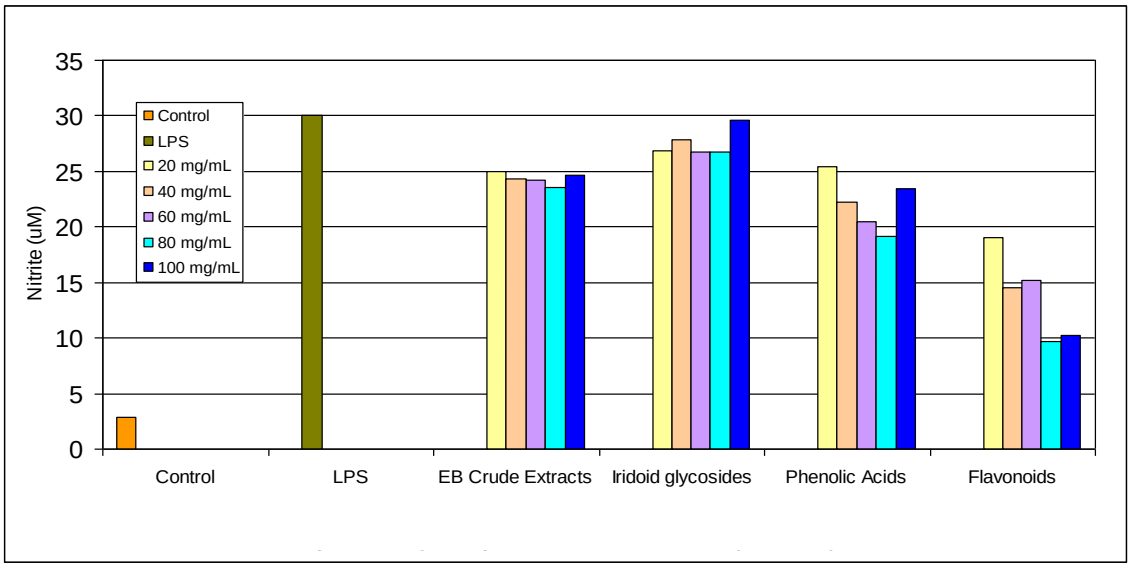


Figure 52. Anti-inflammatory activity of eyebright extracts (crude extracts, iridoid glycoside, phenolic acids, flavonoids).

5 CONCLUSIONS

In this investigation, high-performance liquid chromatography coupled with ultraviolet and electrospray ionization mass spectrometer (HPLC/UV/MS) was used to characterize the chemical constituents in the aerial part of the medicinal plant, Eyebright. Under optimized conditions, the aqueous methanol extract of eyebright was chemically profiled and a total of 28 compounds were identified by interpretation of their UV and MS data, and also by comparison with several standards.Normal column chromatography on polyamide and LC-ESI/MS systems were applied to the phytochemical study of eyebright. Three bioactive fractions were obtained, including total iridoid glycosides, total phenolic acids and total flavonoids. The results of antioxidant and anti-inflammatory investigations showed that both phenolic acids and flavonoids fractions possessed

significant antioxidant properties and that the flavonoids fraction exhibited the most promising anti-inflammatory activities. The iridoid glycosides fraction was further fractionated led to the purification and identification five iridoids glycosides: strictoloside, gardoside methyl ester, ipolamiide, 7-epi-loganin and mussaenoside with chromatographic and spectrometric methods. Other fractions are being purified using a bioassay-directed fractionation utilizing antioxidant and anti-inflammatory assays and LC/MS.

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