12-2010

Extraction and Determination of a Selected Polyphenol from Selected Red and Black Grapes By High Performance Liquid Chromatography.

Summer Nicole Niles
East Tennessee State University

Extraction and Determination of a Selected Polyphenol from Selected Red and Black Grapes By High Performance Liquid Chromatography

Thesis submitted in partial fulfillment of Honors

By

Summer Niles
The Honors College
Midway Honors Program
East Tennessee State University

May 3, 2010

_____
Chu-Ngi Ho, Faculty Mentor

Introduction

Polyphenols are a group of compounds found naturally in plants and they provide much of the flavor, color, and taste to fruits, vegetables, seeds, and other parts of the plants (1). They also act as antioxidants which provide numerous health benefits by protecting cells against damage caused by free radicals. Their antibacterial, anti-inflammatory and anti-allergenic properties have also contributed to the prevention of degenerative diseases, such as cardiovascular diseases and cancers by slowing the rate of oxidative stress on cells (2).

Polyphenols are also found in a wide variety of phytonutrient-bearing foods such as apples, grapes, wines, strawberries, raspberries, blueberries, cranberries, onions, and certain nuts (3). Interestingly, they also are found in coffee and even chocolate (4).

Polyphenols and their Benefits

Researchers have shown that polyphenols from grapes, particularly from their active extraction components such as the seeds, skin, and juice, may be beneficial in preventing some inflammatory-mediated diseases (5), including cardiovascular disease, which is the leading cause of death in the United States (6). The most common polyphenols found in the extracted components of grapes have been identified as resveratrol, phenolic acids, anthocyanins, and flavonoids. They contribute to prevention of disease by decreasing cholesterol oxidation and platelet aggression (6). Resveratrol is found in grape skins as well as in red wine (7). Its structure is shown in Figure 1.

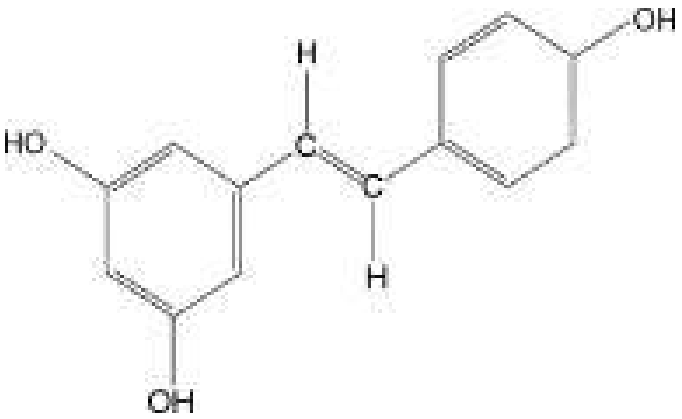
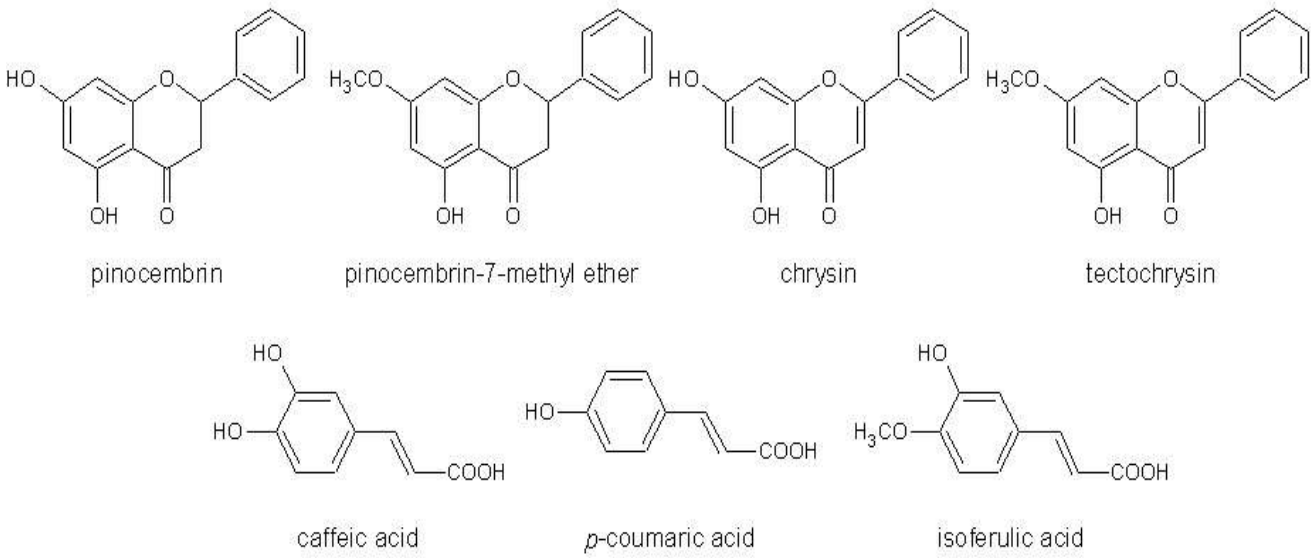


Figure 1: Structure of Resveratrol

Phenolic acids are found in many fruits and vegetables including potatoes, red cabbage, and carrots (8). The different phenolic acids commonly found are shown in Figure 2.

Figure 2: Structures of Some Common Phenolic Acids



Anthocyanins are also found in many fruits and vegetables, such as apples and tomatoes and are the most important group of visible plant pigments besides chlorophyll (9). The chemical structures of some common anthocyanins are shown in Figure 3.

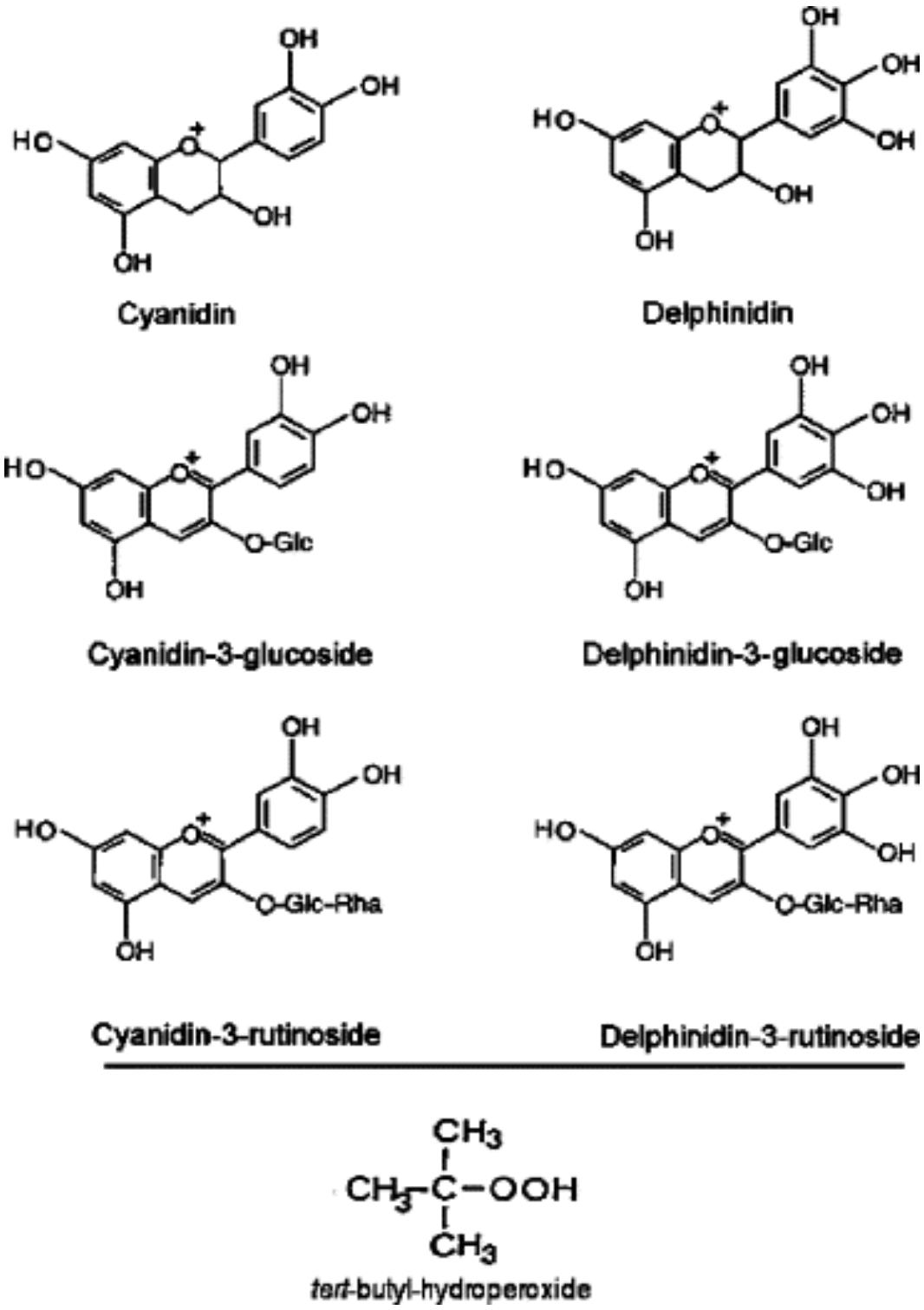


Figure 3: Structures of Some Common Anthocyanins.

In addition to fruits and vegetables, flavonoids are also found in cooking oil, such as canola oil (10). Their chemical structures are shown in Figure 4.

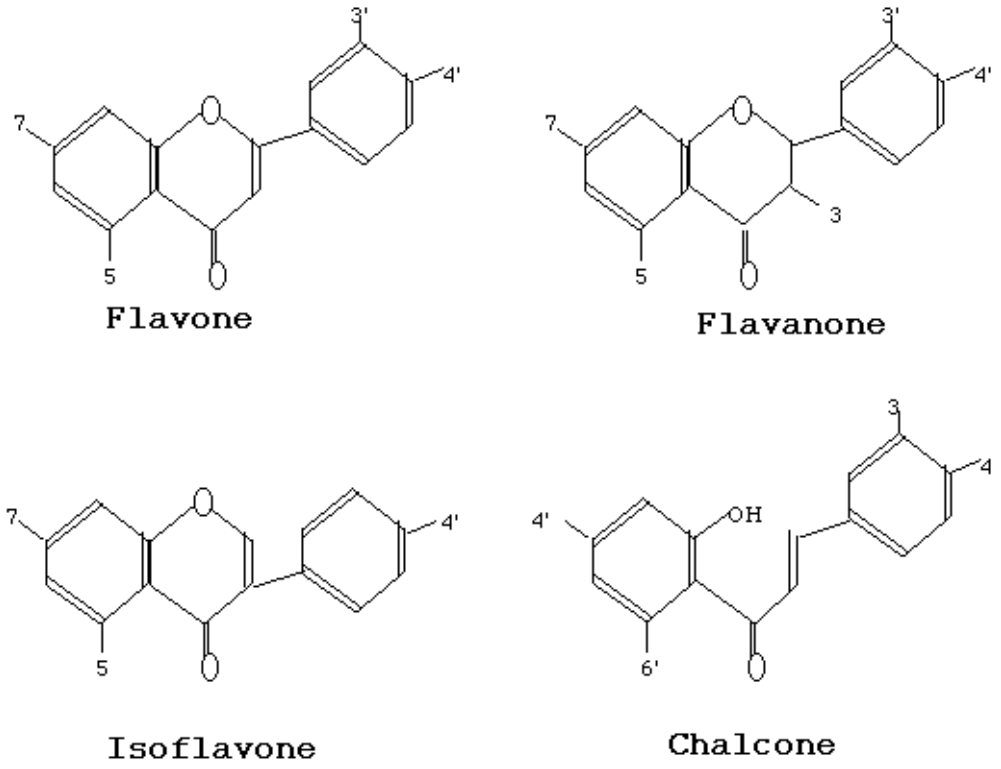


Figure 4: Structures of Some Common Flavonoids

Polyphenols are also associated with the prevention of certain cancers. Every year, approximately 29,000 men die of prostate cancer (11). However, research from clinical trials shows that polyphenols extracted from green tea may play a substantial role in prostate cancer chemoprevention, specifically epigallocatechin-3-gallate (11). Epigallocatechin-3-gallate is also known as EGCG and has been shown to decrease cell viability and promote apoptosis in prostate cancer cell lines while having a neutral effect on healthy cell lines. Green tea also contains epicatechin, epicatechin gallate, and epigallocatechin (11). Some polyphenols found in tea are shown in Figure 5.

Additionally, tea polyphenols have been shown to exhibit preventive effects against skin, liver, lung, gastrointestinal tract, pancreatic, and bladder cancer in laboratory animals (12).

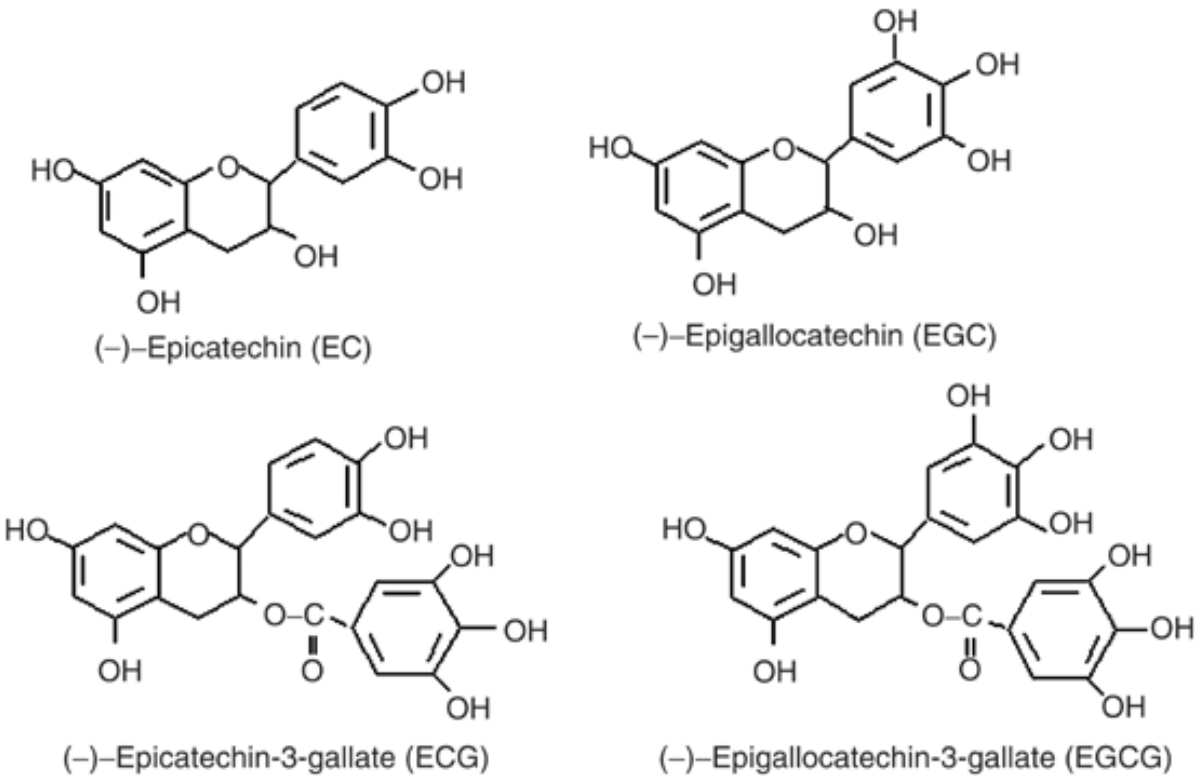


Figure 5: Structures of Some Common Tea Polyphenols

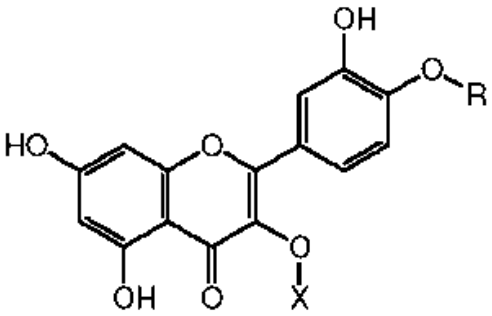
Catechins extracted from green tea have shown to have a preventive effect on Parkinson disease which is thought to be caused by oxidative stress, particularly on dopaminergic neurons (13). Furthermore, there is a strong evidence to support that green tea catechins may play a potential role in the treatment of Alzheimer's disease due to their inhibitory and neuroprotective effects (13). A research study (14) also shows that EGCG may afford protection against stroke by guarding against cerebral ischemic damage.

Furthermore, according to the Centers for Disease Control and Prevention, 7.8% of the population in the United States, or over 23 million people, suffer from diabetes (15). However, one small study of human volunteers (16) found that catechins from green tea substantially increased oral glucose tolerance but did not affect basal blood

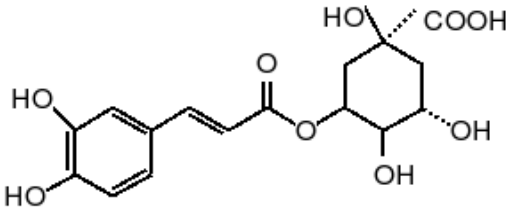
glucose levels. These results were shown to be very useful since insulin resistance and glucose intolerance are two key features of type II diabetes. A study performed in normal lab rats (17) also showed that polyphenolic extracts from green tea increased insulin sensitivity after long-term administration. Additionally, a similar study (18) found that polyphenolic extracts from green tea prevented the development of insulin resistance, hyperglycemia, and other metabolic defects when administered to fructose-fed lab rats. These results show the potential of polyphenolic extracts and their positive effects on diabetes.

Several research studies also suggest that oral consumption of polyphenols may protect against obesity-related disorders, such as arthrosclerosis and hypertension (8). Statistical data from the Centers for Disease Control and Prevention estimated that nearly one third of all American adults are obese (19). However, one interesting study showed that purified EGCG significantly reduced and prevented an increase in body weight in both lean and obese Zucker rats (20). This effect also appeared to be reversible and was associated with a reduction of food intake which demonstrates the possibilities of consuming foods rich in polyphenols for treating obesity.

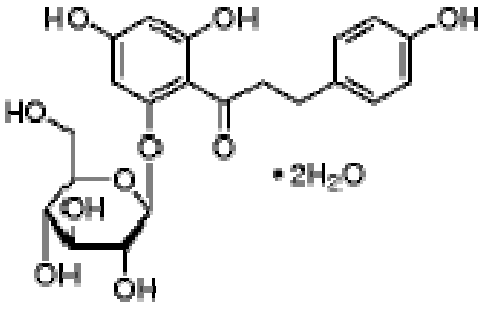
Polyphenols extracted from apples peels have also shown to contribute to the prevention of asthma and have been positively associated with general pulmonary health (21). These include procyanidins, catechin, epicatchin, chlorogenic acid, phloridzin, and quercetin (21). The structures of these compounds are shown in Figure 6.



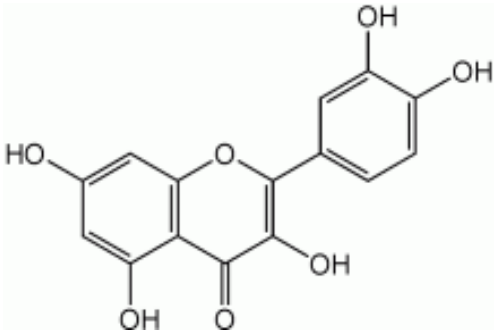
Procyanidin



Chlorogenic Acid



Phloridzin



Quercetin

Figure 6: Structures of Some Important Polyphenols

One study involving 1600 adults in Australia found that apple and pear consumption was associated with a decrease in asthma and a decrease in bronchial sensitivity due to antioxidant activity from polyphenols (22). A similar study consisting of 13,000 adults in the Netherlands (23) found that catechin intake from both apples and pears was associated with pulmonary function and negatively associated with chronic obstructive pulmonary disease.

Research results have demonstrated the numerous contributions that polyphenols make toward public health, particularly by prevention of degenerative diseases and cancers. These results also aid in showing how a diet that is rich in fruits

and vegetables, which contain large amounts of polyphenols, is greatly beneficial to human health.

Analytical Methods for Analysis

Extraction Techniques

The most common method for the extraction of polyphenols from fruits is organic solvent extraction and concentration. In organic solvent extraction, an organic solvent such as ethanol or methanol is used to extract the polyphenols from the fruit or vegetable that is being studied. The same solvent is then used for making the concentrated solutions in a volumetric flask. Nawaz et al. (24) used this method for the extraction of polyphenols from grape seeds. Approximately 40 grams of milled grape seed was mixed for five minutes with 200 mL of a 50% ethanol solution. The solution was then extracted in the dark for one hour. The top phase was then filtered through Watman filter paper #4. The level of total polyphenols was determined using Folin–Ciocalteau colorimetric reaction method. Under these conditions, the maximum amounts of polyphenols were recovered from the grape seeds which consisted of 11.4% of the total weight of the grape seeds.

Bucic-Kojik et al. (25) performed triplicate extractions of polyphenols from milled grape seed using 50% ethanol as a solvent to determine the kinetics. Different solid-liquid ratios were created by mixing 0.5 g of the sample with 5 mL, 10 mL, 15 mL, and 20 mL of solvent, respectively. The test tubes were then incubated in a water bath at different temperatures ranging from 25°C to 80°C for two hours. They were shaken for around 20 seconds at 15 minute intervals by Vortex. Using this technique, the highest

extraction yield was obtained at a solid-liquid ratio of 40 mL/g. This ratio was then used for kinetic study at different extraction temperatures which resulted in the higher temperatures yielding the largest polyphenol contents. The extracts were then decanted and centrifuged for five minutes. The supernatants were diluted to 25 mL using the 50% aqueous ethanol. The concentration of total polyphenols in the extracts was then determined by spectrophotometric Folin-Ciocalteau method which yielded approximately 80-90% of the total polyphenols, thus making this an acceptable method for polyphenol extraction

Bousetta et al. (26) used a high voltage electrical discharges (HVED) method for the extraction of polyphenols from grape pomace. HVED were applied to fresh, frozen-thawed and sulphured grape pomaces for 160 seconds and diffusion was then studied for one hour. Sulphur dioxide was used for the preservation and was found to be better than freezing. The results showed that after 40 minutes, the yield of extracted solutes from fresh grape pomace reached 70% which represented more than twice the yield obtained after 240 minutes with HVED. HVED was also shown to increase the yield of polyphenols after one hour of extraction when compared to that obtained after 4 hours of extraction without HVED. Furthermore, the polyphenol yields increased with temperature. Overall, the results show that HVED greatly increases the total yield of polyphenols extracted.

High Performance Liquid Chromatography

Once the polyphenols are extracted, High Performance Liquid Chromatography (HPLC) can be used to identify, separate, and quantify the different polyphenol

compounds based on their idiosyncratic polarities and interactions with the column's stationary phase. Due to its usefulness, several researchers have used it in previous studies for both separation and quantitation of grape polyphenols.

Pascual-Marti et al. (27) used HPLC with UV detection at 306 nm to determine the resveratrol content in ethanolic extracts of different grape skins. Acetic acid: methanol: water 5:20:75 was used as the mobile phase and twenty microliters of sample was used as the injection volume. The analysis was also performed at room temperature using six different samples. The resveratrol content was established using two columns, C-18 and C-8. The flow rates of 1.0 and 1.5 mL per minute were also selected for both columns. The results showed that C-18 stationary phase provided a better precision but a poorer sensitivity and smaller linear interval. The highest reported content of resveratrol found in the grape skin extracts using this method was 170.6 mg of resveratrol per 1 kg of grape skin.

Huang et al. (28) used HPLC -MS with electrospray ionization for the analysis of anthocyanins in different cultivars of muscadine grapes. The mobile phase consisted of a 5:95 formic acid: water solution in phase A and a 5:95 formic acid:methanol solution in phase B at a flow rate of 0.8 mL per minute using a C-18 column. The linear gradient of phase B was 15% for the first two minutes which then increased to 45% up until 30 minutes, and decreased to 15% for one more minute. The 15% phase B was maintained up to 40 minutes. The UV-Vis detector was set to 520 nm and mass spectra were acquired in positive ion mode. Ion was scanned from 200 to 700 m/z with a scan speed of 1000 amu per second. Nitrogen was also used as a nebulizing and drying gas. The nebulizing gas flow was 1.5 L per minute and the drying gas pressure was 0.1

MPa. The individual anthocyanins were then identified based on retention times and molecular weight. Later they were quantified using cyanidin chloride as an internal standard. The highest reported amount of anthocyanins using this method was 4.1 mg per gram of fresh grapes, and this was found in the Jumbo' cultivar.

Andrade et al. (29) used a solid-phase extraction and HPLC method to determine differences in flavanol compositions in seven varieties of port wine grapes. The mobile phase consisted of a solution of 19:1 water: formic acid in phase A and 60% methanol in phase B at a flow rate of 0.3 mL per minute. Detection was achieved using a diode array detector and chromatograms were recorded at 280 and 350 nm. The different compounds in each sample were identified by comparing their retention times and UV-vis spectra, in the 200-400 nm range, with those of standards. Quantitation was achieved by comparing the absorbances recorded in the chromatograms to those of internal standards of phenolics. Catechin, epicatechin, and syringic acid were detected at 280 nm while others were detected at 350 nm. Epicatechin was found to be the major compound, although all samples showed significant amounts of kaempferol 3-glucoside and isorhamnetin 3-glucoside. Furthermore, the *Touriga Nacional* variety of grapes contained the highest amount of kaempferol 3-glucoside which was 77.3 mg per kg while the Tinta Barroca variety contained the highest amount of isorhamnetin 3-glucoside which was 139 mg per kg.

Palomino et al. (30) used reversed-phase high-performance liquid chromatography to test several polyphenols in grape berries from Spain. Using this method, five polyphenols were determined and quantified which included cis- and trans-resveratrol, quercetine, quercitrine, and rutine. Reference substances were purchased

and several aliquots of the solutions of every standard were diluted in order to obtain reference solutions of decreasing concentration. The reference standards were then subjected to analysis using the following experimental conditions: pump A, acetonitrile; pump B, water-acetic-acid-acetonitrile (87:3:10) in a linear gradient elution of 5% A and 95% B starting at 0 minutes and 25% A and 75% B after 25 minutes. The flow rate was 1.0 mL per minute and detection was set to 306 nm. The injection volume was 10 µL and the column used was a Hypersil ODS 150 x 4.6 mm, 5µm. T_0 was also calculated by three successive injections of a KNO_3 solution in 100% methanol which resulted in a value of 1.05 minutes.

The standard solutions were then further analyzed by plotting their corresponding peak areas against the concentration of polyphenols injected. The polyphenol concentrations in the samples were calculated from the chromatogram peak areas using the normalization method. Furthermore, the identification of the different compounds was achieved by comparing both the retention time and the absorption spectra obtained for each eluted peak with those obtained for the standards. The highest reported contents of rutine, quercitrine, trans-resveratrol, quercetine were as follows: 2.56 mg/100 g, 5.4 mg/100 g, 9.6 µg/100 g, and 0.17 mg/100 g of sample. Cis-resveratrol was not found in any of the samples. The results showed that the recovery of the polyphenols in the grape berries was 94% or higher and the sensitivity of the method was also found to be very good, thus making this an excellent method for the determination and quantification of polyphenols.

Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance Spectroscopy, commonly referred to as NMR is a general technique for determining the structure of organic compounds. It involves the absorption of radiation by nuclei in organic molecules in a strong electromagnetic field (31) and can be used to compliment HPLC for the analysis and identification of polyphenols.

Lu et al. (32) used NMR spectroscopy after HPLC to identify 17 polyphenols in Chardonnay grape pomace. This study also reported gallic acid 3-β-glucopyranoside, gallic acid 4-β-glucopyranoside and 2-hydroxy-5-(2-hydroxyethyl)phenyl-β-glucopyranoside as natural grape constituents for the first time which demonstrates that this was a very effective method.

Liquid Chromatography-Mass Spectrometry

Liquid Chromatography-Mass Spectrometry (LC/MS) is a powerful technique often used in analytical chemistry due to its high sensitivity and specificity. It can also be used in polyphenol research to detect and identify specific polyphenolic compounds in the presence of other chemicals in a complex mixture.

Borbalan et al. (33) used LC/MS to determine the polyphenol content of six varieties of red and white grapes. For the separation of the compounds, the chromatographic conditions consisted of a flow rate of 0.15 mL per minute, sample injection volume of 20 µL and mobile phases of 1% methanol, 1% acetic acid, 98% water in phase A and 90% Methanol, 2% acetic acid, 8% water in phase B. The inferface conditions were as follows: negative ionization mode, 250 °C capillary

temperature, 4.5 kV spray voltage, -5 V capillary voltage, focus gas flow of 80, and an auxiliary gas flow of 10. The mass detection was performed between 100 and 1000 m/z. An activation energy of 25% was also applied. Nine phenolic compounds were clearly identified. These included catechin, epicatechin, epicatechingallate, and caftaric acid. Further analysis using HPLC concluded that catechin had the highest content in both red and white grapes. The results show that LC/MS is a good method for the identification of compounds, but is not the best method for the quantification of phenolic compounds. HPLC is needed for this portion of the research.

Research Objectives

From previous discussions, this study is geared toward extracting and establishing the quantities of a specific polyphenol found in different varieties of grapes: quercetin. The different varieties include red and black seedless grapes. The research objectives are given below:

1. To develop an efficient extraction method to be used for analysis.
2. To develop an efficient HPLC procedure for the determination and quantitation of quercetin for the two different grape varieties.
3. To compare percentage amounts of the polyphenol in both varieties of grapes.

Experimental Procedure

In the previous discussion, methods that have been used to extract and quantify polyphenols were discussed. The following includes a detailed description of the procedure that was used for the analysis of quercetin in two varieties of selected grapes. We shall begin with discussing the samples and the reagents that were used in the study and how each one of them was prepared for the HPLC analysis. Thereafter, the HPLC system used and how basic conditions for the analysis such as wavelength, mobile phase, and flow rate were optimized is described.

Preparation of Samples and Standards

Initially, red and black seedless grapes were purchased from Kroger© in Johnson City, TN. Six 100-gram samples of red grapes and six 100-gram samples of black grapes were then chopped and prepared for extraction using a 95% ethanol solution. Approximately 60 mL of the 95% ethanol solution was poured onto the chopped grape samples. The samples were mixed for five minutes before being placed into a sonicator to extract the polyphenols in the dark for one hour. The samples were then filtered once using vacuum filtration. A 150-mL filter flask and type II Whatman filter paper were used in this procedure. The filter flask was rinsed twice with 10 mL of the 95% ethanol solution to ensure that the maximum amount of polyphenols was obtained. The sample solutions were then placed inside 100-mL volumetric flasks and filled to the mark with the 95% ethanol solution. The 100-mL sample solutions were then transferred into dark bottles and stored in the freezer until analysis. Quercetin was also purchased to be used as a standard for analysis. Two hundred milligrams of quercetin were weighed on an analytical balance and was dissolved in 100 mL of the 95% ethanol solution in a

volumetric flask. Solutions of 1 mg/mL, 0.4 mg/mL, and 0.2 mg/mL of quercetin were prepared from the initial 2 mg/mL quercetin solution for quantitative analysis.

HPLC Parameters

The mobile phase used for HPLC analysis was a 5:15:80 glacial acetic acid:methanol: water solution. This solution was prepared by adding 50 mL of glacial acetic acid, 150 mL HPLC grade methanol, and 800 mL of water in a 1000 mL-beaker. Helium was then passed into solution for twenty minutes to degass the solution. The stationary phase was a C-18 column. The injection volume was 20 µL and the flow rate was 1.0 mL per minute. UV detection was initially set to 255 nm and was later changed to 306 nm. The run time was initially set to thirty minutes. It was eventually changed to ten minutes and then to six minutes after determining that the retention time of quercetin was around five minutes. Turbochrom software from Perkin Elmer was then used for analysis. Each quercetin standard solution was run twice and the peak areas and retention time were recorded. Following this, triplicate samples of each grape extract were run and their peak areas and retention times were also recorded. Quercetin was identified in the samples by comparing retention times of eluted peaks with that of the quercetin standard. Quantitation of quercetin was then performed by creating a calibration curve using the different quercetin standard solutions. By plotting the different concentrations against the areas under the peaks, a linear regression line could be obtained. Using the equation of the best fit line, the concentration of quercetin in each sample was calculated, along with the mean concentration and relative standard deviation.

Results

The HPLC results obtained are tabulated in Table 1 and Table 2. The retention time tabulated is that of the peak identified to be that of quercetin. The peak areas of the corresponding peaks are also given in the tables.

Table 1: HPLC data for Quercetin standard solutions

Quercetin Standard	Retention Time (minutes)	Area (UV^{α} sec)
2 mg/mL	4.53	204,934.3
2 mg/mL	4.47	29,149.4
2 mg/mL	4.50	172, 225.6
2 mg/mL	4.54	114, 922.9
1 mg/mL	4.51	121,748.4
1 mg/mL	4.50	153,039.4
1 mg/mL	4.44	72,351.1

Table 2: HPLC data for samples of red and black grapes

Sample	Retention Time (minutes)	Area (UV^{α} sec)
Red 6	4.60	99,388.9
Red 5	4.47	93,903.3
Red 5	4.67	196, 676.5
Red 5	4.52	16,629.0
Black 4	4.41	204,934.3
Black 4	4.70	29,149.4

Black 4	4.42	172,225.6

Because of the HPLC malfunction and since no HPLC instruments were available to us to make more measurements, a calibration curve could not be obtained. Also as shown from the data tabulated in Table 1, one of the HPLC runs (the second one) was definitely in error, and the others were not as reproducible. Hence the accurate determination of the amount of quercetin in the grape samples was not possible. Only estimates could be made about their amounts from the data shown in Table 2. These estimates were shown in Table 3. The important conclusion is that these grapes sample do contain a reasonably high amount of quercetin.

Table 3: Average Concentration of Quercetin in the grape samples

Grape Sample	Average Concentration of Quercetin (mg/mL)
Red 6	5.82
Red 5	1.54
Black 4	8.29

The chromatograms of the different grape samples are shown in Figure 7 to Figure 13. As shown in these chromatograms, the quercetin peak has not been well resolved using the mobile phase and detection wavelength used. Unfortunately, we had not been able to optimize the chromatographic conditions due to instrumental problems encountered. Figure 14 shows the chromatogram of the quercetin standard and it gave us the retention time of the compound with which we used to identify the quercetin in the sample chromatograms.

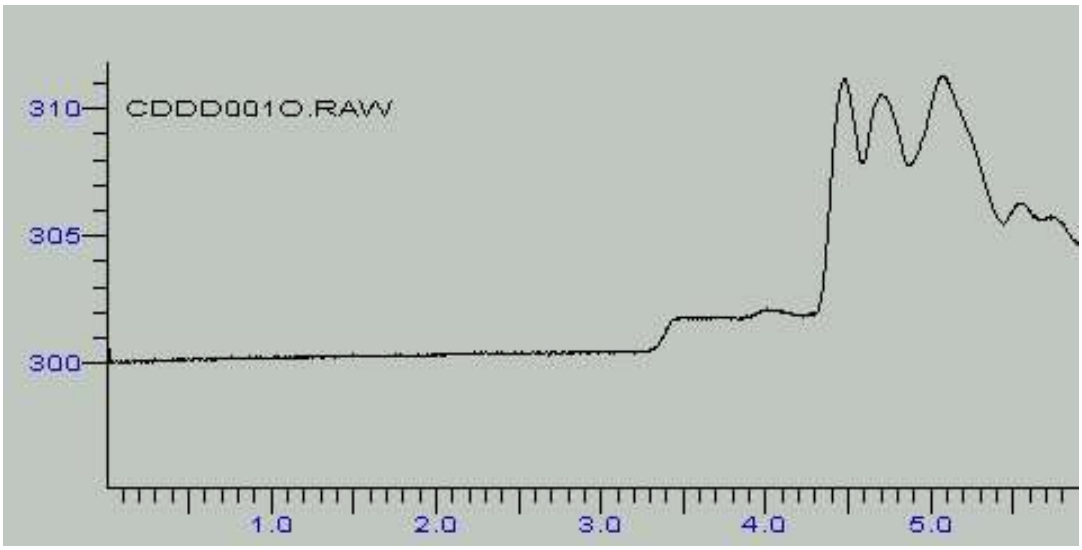


Figure 7: Chromatogram of Red Grape Extract #5 Run #1

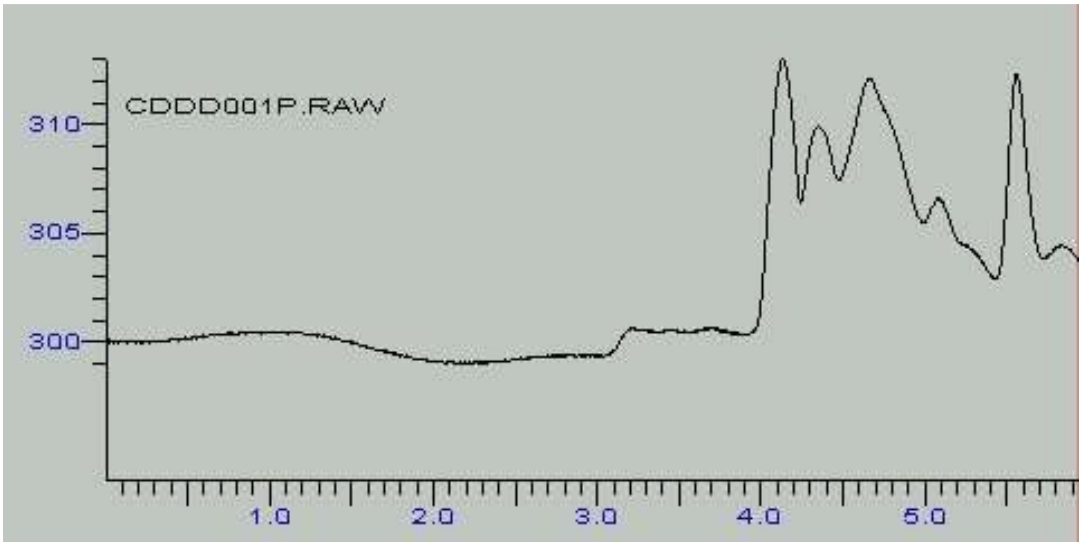


Figure 8: Chromatogram of Red Grape Extract #5 Run #2

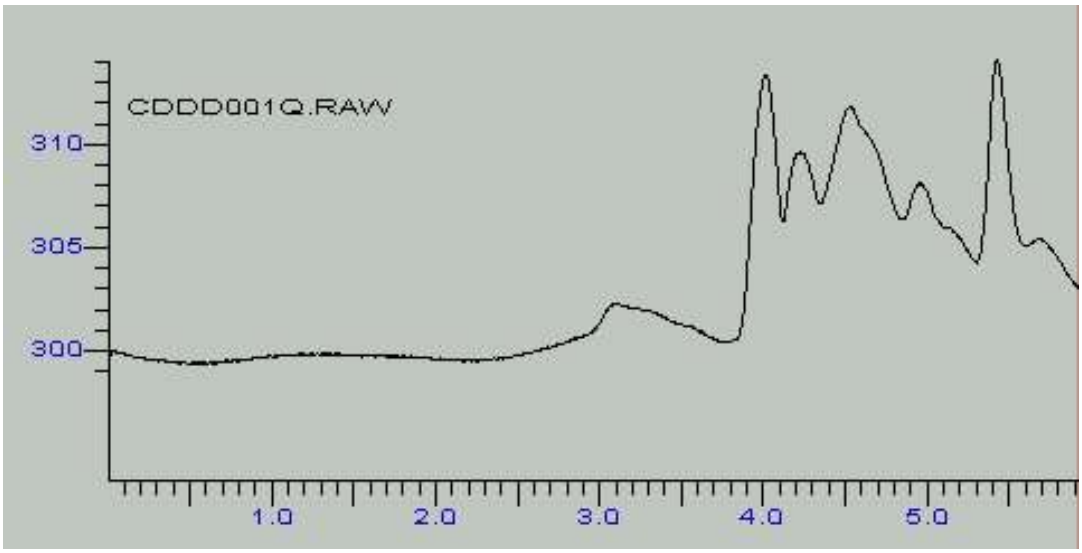


Figure 9: Chromatogram of Red Grape Extract #5 Run #3

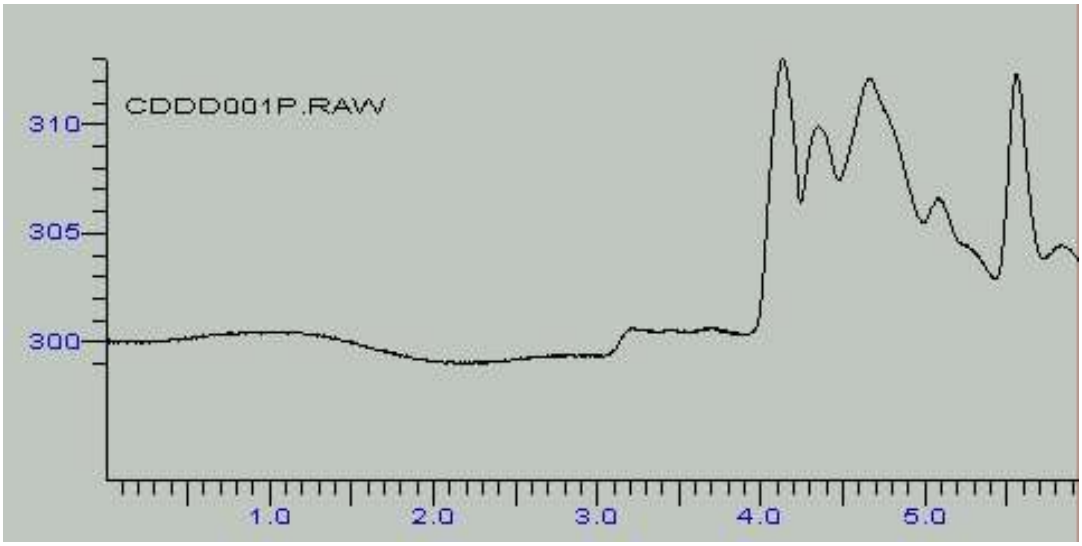


Figure 10: Chromatogram of Red Grape Extract #6

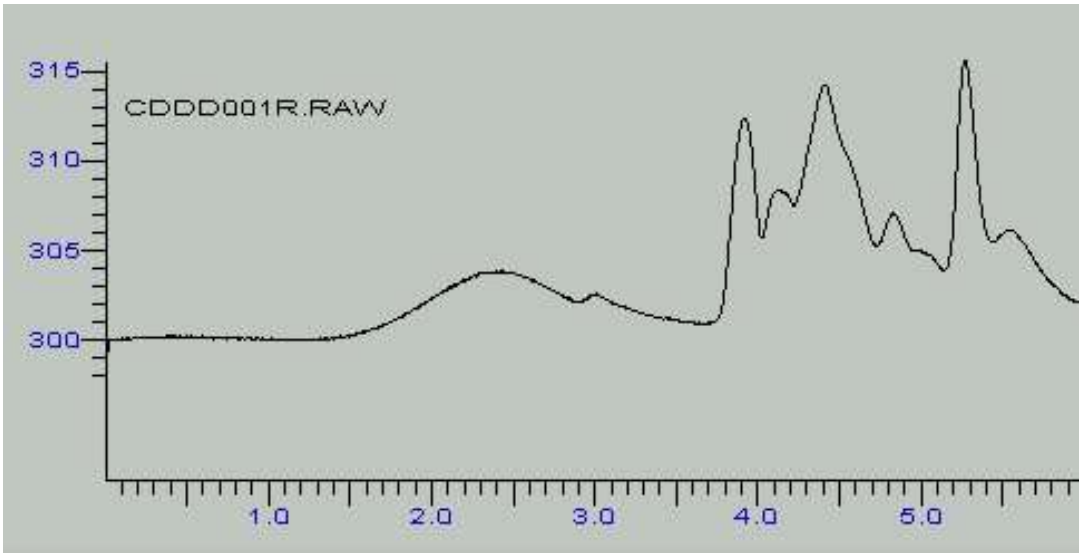


Figure 11: Chromatogram of Black Grape Extract #4 Run #1

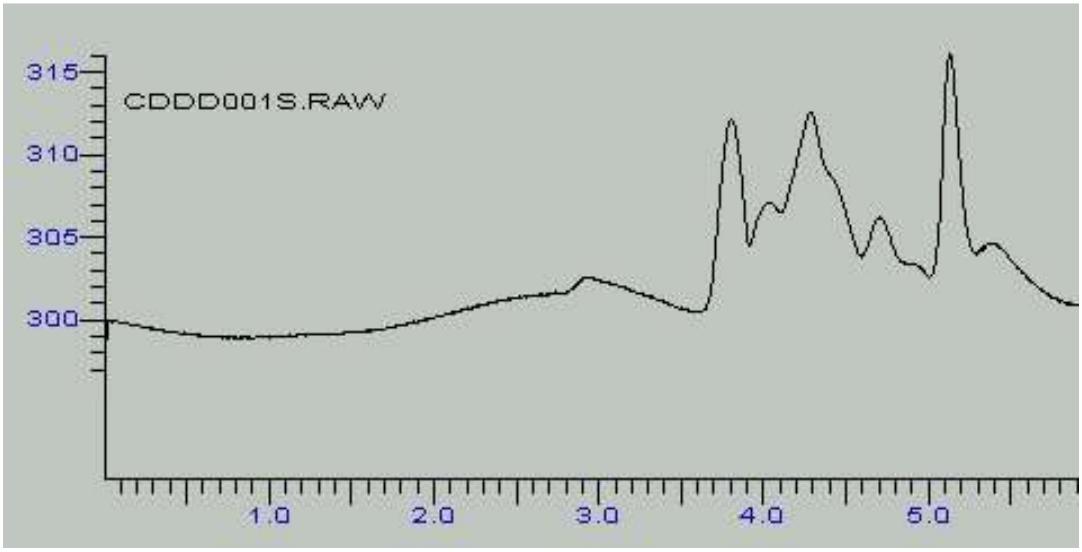


Figure 12: Chromatogram of Black Grape Extract #4 Run #2

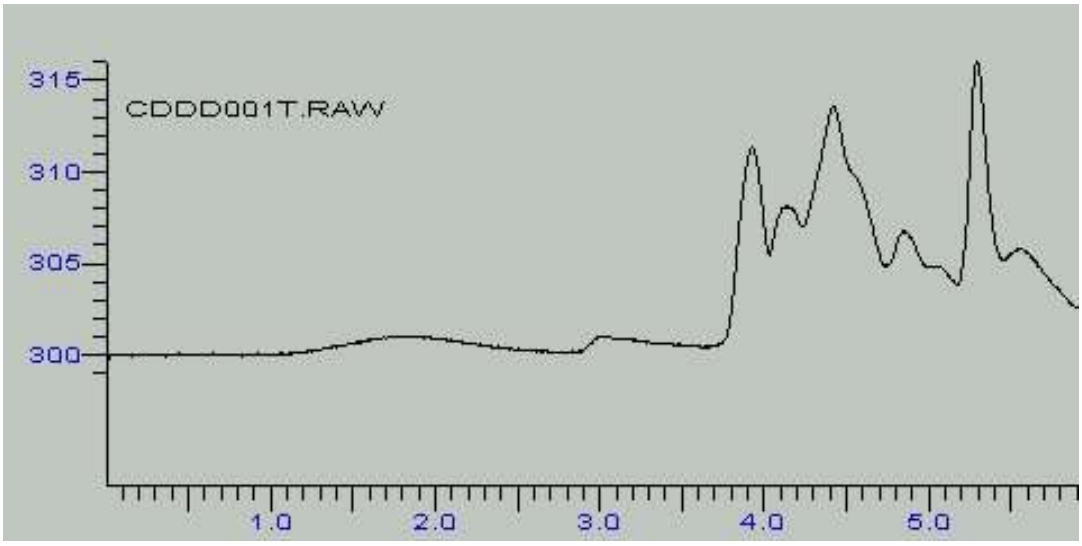


Figure 13: Chromatogram of Black Grape Extract #4 Run #3

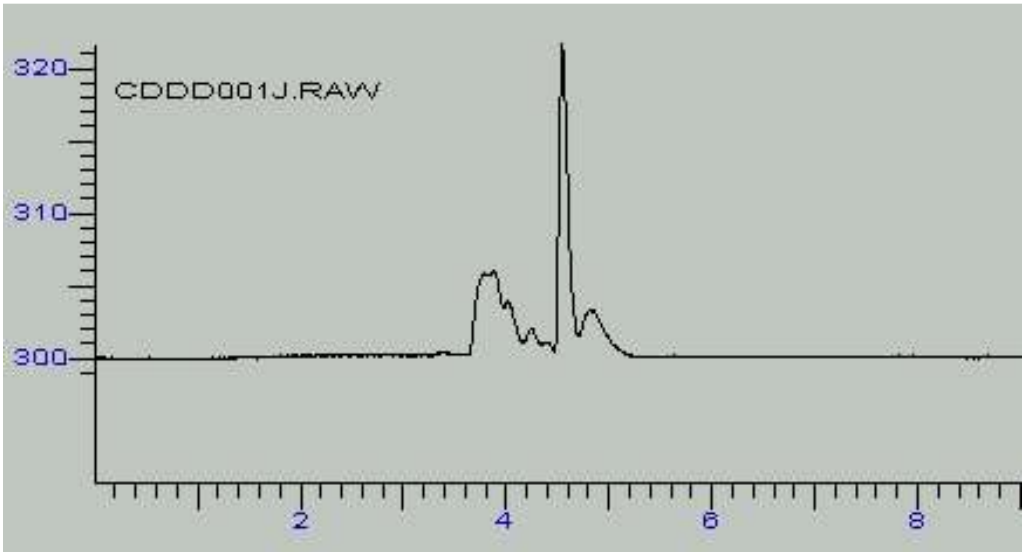


Figure 14: Chromatogram of quercetin standard

Discussion of Results

Triplicate runs were performed for red grape extract #5 and black grape extract #6 to ensure more accurate and precise results. As previously mentioned, the retention

times along with the areas for the eluted peaks of quercetin found in these samples are shown in Table 2 using the retention time of quercetin standards shown in Table 1. The average retention time for the eluted peak for quercetin in red grape extract #5 was calculated to be 4.55 minutes. The average retention time for the eluted peak for quercetin in black grape extract #4 was calculated to be 4.51 minutes. The average area under the eluted peak of quercetin in red grape extract #5 was calculated to be 1.02×10^5 UV^{α} sec and that of black grape extract #4 was calculated to be 1.35×10^5 UV^{α} sec. Red grape extract #6 was also run once through HPLC and its data is recorded in Table 2. Prior to these sample runs, quercetin standard solutions of 2 mg/mL and 1 mg/mL concentrations were analyzed using HPLC under identical chromatographic conditions. Their retention times and areas are recorded in Table 1. Because an insufficient amount of data was obtained prior to instrumental failure, a calibration curve could not be made, since only data for the concentrations of 2 mg/mL and 1 mg/mL of quercetin were available. In this case, they were used for the estimation of the concentration of quercetin in the samples rather than for the exact determination. Additional runs of standards of decreasing concentration were needed in order to obtain more accurate and precise results for the samples. Although the results obtained are not very accurate, they do show that the black grape sample had a higher concentration of quercetin present in the sample than in the red grape sample. This observation is consistent with my hypothesis that the black grape samples would contain a greater concentration of quercetin than the red grape samples due to the black grapes' deep dark color. Furthermore, the chromatograms for each sample subjected to hplc are also previously listed in the results section. Above this section is a

chromatogram of a solution containing quercetin that was obtained at a wavelength of 306 nm using the same mobile phase of water: methanol: glacial acetic acid as the previous samples and standards.

This chromatogram clearly shows the retention time of the eluted peak of quercetin which was at 4.54 minutes. The eluted peak area was around 150,000 UV^{α} sec. Furthermore, the six other quercetin standard chromatograms appeared to be visually similar to this chromatogram which shows that they were fairly consistent with one another.

The reason for the lack of results for the rest of the grape sample extracts is due to technical problems with the HPLC instrument, specifically power supply issues. Future research should be directed at analyzing triplicate runs of every grape extract sample and analyzing different concentration solutions of the quercetin standard. After obtaining this data, a calibration curve should be constructed in order to obtain a line of best fit. Once this best fit line is obtained, it should be used to calculate the concentration of quercetin in each sample. The average concentration and relative standard deviation can then be calculated.

Conclusion

Polyphenols play an important role in human health as antioxidants in many fruits and vegetables. Their antibacterial, anti-inflammatory and anti-allergenic properties have also contributed to the prevention of degenerative diseases and numerous cancers.

Organic solvent extraction using methanol proved to be an effective method for extracting the polyphenols out of the selected grapes. Furthermore, even though technical problems arose from the HPLC instrument, HPLC still proved to be an effective method for the separation and quantitation of the compounds extracted with the highest estimated average yield being 8.29 mg/mL of quercetin in black grape extract #4.

Future research is needed to obtain more accurate and reliable results. This would include analyzing triplicate runs of every grape extract sample and different concentration solutions of the quercetin standard. Further analysis can then conducted upon obtaining this data which includes quantitation of quercetin in each sample and calculating relative standard deviations.

References

1. Polyphenolics. Polyphenol Health Supplies. http://www.meganatural-bp.com/polyphenolics/
2. Johnson, I. T.; Saltmarsh, M.; Scalbert, A. Polyphenols: Antioxidants and Beyond, *The American Journal of Clinical Nutrition.* **2005**, 81, pp 215-217.
3. United State Department of Agriculture. Agricultural Research Services. http://www.ars.usda.gov/Aboutus/docs.htm?docid=4142
4. Wollgast, Jan; Anklam, Elke. Polyphenols in Chocolate: Is there a Contribution to Human Health, *Food Research International*. **2000**, 33, pp 449-459.

5. Leifert, Wayne R.; Abeywardena, Mahinda Y. Grape Seed and Red Wine Polyphenol Extracts Inhibit Cellular Cholesterol Uptake, Cell Proliferation, 5-Lipoxygenase Activity, *Nutrition Research*. **2008**, 28, pp 842-850.

6. Abeywardena, Mahinda Y.; Leifert, Wayne R. Cardioprotective Actions of Grape Polyphenols, *Nutrition Research*. **2008**, 28, pp 729-737.

7. National Cancer Institute. U.S. National Institutes of Health. http://www.cancer.gov/cancertopics/factsheet/prevention/redwine

8. Hellstrom, Jarkko; Mattila, Pirjo. Phenolic Acids in Potatoes, Vegetables, and Some of their Products, *Journal of Food Composition and Analysis*. **2007**, 20, pp 152-160.

9. Brouillard, R.; Chia, Lian-Sai; Chia, Tet-Fatt; Goh, Ngoh-Khang; Kong, Jin-Ming. Analysis and Biological Activities of Anthocyanins, *Phytochemistry*, **2003**, 64, pp 923-933.

10. Chan, P.T.; Chen, Z.Y.; Fung, K.P.; Ho, K.Y.; Wang, J. Antioxidant Activity of Natural Flavonoids is Governed by Number and Location of their Aromatic Hydroxyl Groups, *Chemistry and Physics of Lipids*, **1996**, 79, pp 157-163.

11. Bailey, H.H.; Johnson, J.J.; Mukhtar, H. Green Tea Polyphenols for Prostate Cancer Chemoprevention: A Translational Perspective, *Phytomedicine*, **2010**, 17, pp 3-13.

12. Khan, Naghma; Mukhtar, Hasan. Tea Polyphenols for Health Promotion, Life Sciences, **2007**, 81, pp 519-533.

13. Zaveri, Nurulain T. Green Tea and its Polyphenolic catechins: Medicinal Uses in Cancer and Noncancer Applications, *Life Sciences*, **2006**, 78, pp 2073-2080.

14. Ikeda, K.; Suzuki, M.; Tabuchi, M.; Tomita, T.; Umegaki, K. Protective Effects of Green Tea Catechins on Cerebral Ischemic Damage, *Medical Science Monitor*, **2006**, 10, pp 166-174.

15. Centers for Disease Control and Prevention. Diabetes Public Health Resource: Diabetes Statistics and Research. http://www.cdc.gov/diabetes/faq/research.htm (accessed Feb 12, 2010).

16. Tsuneki, M.; Ishizuka, M.; Wu, J.B.; Sasaoka, T.; Kimura, I. Effect of Green Tea on Blood Glucose Levels and Serum Proteomic Patterns in Diabetic (DB/DB) Mice and on Glucose Metabolism in Healthy Humans, *BMC Pharmacology*, **2004**, 4, p 18.

17. Wu, L.Y.; Juan, C.C.; Ho, L.T.; Hsu, Y.P.; Hwang, L.S.; Effect of Green Tea Supplementation on Insulin Sensitivity in Sprague-Dawley Rats, *Journal of Agricultural and Food Chemistry*, **2004**, 52, pp 643-648.

18. Wu, L.Y.; Juan, C.C.; Hwang, L.S.; Hsu, Y.P.; Ho, P.H.; Ho, L.T. Green Tea Supplementation Ameliorates Insulin Resistance and Increases Glucose Transporter IV Content in a Fructose-Fed Rat Model, *European Journal of Nutrition*, **2004**, 43, pp 116-124.

19. Centers for Disease Control and Prevention. Overweight and Obesity: Data and Statistics. http://www.cdc.gov/obesity/data/index.html (accessed Feb 13, 2010).

20. Kao, Y.H.; Hiipakka, R.A.; Liao, S. Modulation of Obesity by a Green Tea Catechin, *American Journal of Clinical Nutrition*, **2000**, 72, pp 1232-1234.

21. Boyer, Jeanelle; Liu, Rui Hai. Apple Phytochemicals and their Health Benefits, *Nutrition Journal*, **2004**, 3, pp 5-19.

22. Woods, R.; Walters, H.; Raven J.; Wolfe, R.; Ireland, P.; Thien, F.; Abramson, M. Food and Nutrient Intakes and Asthma Risk in Young Adults, *American Journal of Clinical Nutrition*, **2003**, 78, pp 414-421.

23. Tabak, C.; Arts, I.; Smit, H.; Heederik, D.; Kromhout, D. Chronic Obstructive Pulmonary Disease and Intake of Catechins, Flavonols, and Flavones*, American Journal of Respiratory Critical Care Medicine*, **2001**, 164, pp 61-64.

24. Nawaz, Haseeb; Shi, John; Mittal, Gauri S.; Kakuda, Yukio. Extraction of Polyphenols from Grape Seeds and Concentration by Ultrafiltration, *Separation and Purification Technology*, **2006**, 48, pp 176-181.

25. Bucic-Kojic, Ana; Planinic, Mirela; Tomas, Srecko; Bilic, Mate; Velic, Darko. Study of Solid-Liquid Extraction Kinetics of Total Polyphenols from Grape Seeds, *Journal of Food Engineering*, 81, **2007**, pp 236-242.

26. Boussetta, Nadia; Lanoisell, Jean-Louis; Bedel-Cloutour, Catherine; Vorobiev, Eugene. Extraction of Soluble Matter from Grape Pomace by High Voltage Electrical Discharges for Polyphenol Recovery: Effect of Sulphur Dioxide and Thermal Treatments, *Journal of Food Engineering*, **2009**, 95, pp 192-198.

27. Pascual-Marti, M.C.; Salvador, A.; Chafer, A.; Berna, A. Supercritical Fluid Extraction of Resveratrol from Grape Skin of *Vitis vinifera* and Determination by HPLC, *Talanta*, **2001**,54, pp 735-740.

28. Huang, Zhiliang; Wang, Baowu; Williams, Paul; Pace, Ralphenia D. Identification of Anthocyanins in Muscadine Grapes with HPLC-ESI-MS, *LWT- Food and Science Technology*, **2009**, 42, pp 819-824.

29. Andrade, P.B.; Mendes, G.; Falco, V.; Valentao, P.; Seabra, R.M. Preliminary Study of Flavonols in Port Wine Grape Varieties, *Food Chemistry*, **2001**, 73, pp 397-399.

30. Palomino, O.; Gomez-Serranillos, M.P.; Slowing, K.; Carretero, E.; Villar, A. Study of Polyphenols in Grape Berries by Reversed-Phase High-Performance Liquid Chromatography, *Journal of Chromatography A*, **2000**, 870, pp 449-451.

31. Edwards, John C. “Principles of NMR”, Process NMR Associates LLC, 1999-2005, http://www.process-nmr.com/nmr.htm

32. Lu, Yinrong; Yeap Foo, L.The polyphenol Constituents of Grape Pomace, *Food Chemistry*, **1999**, 65, pp 1-8.

33. Borbalan, Angeles M. Alonso; Zorro, Luisa; Guillen, Dominico A.; Barroso, Carmelo Garcia. Study of the Polyphenol Content of Red and White Grape Varieties by Liquid Chromatography-Mass Spectrometry and its Relationship to Antioxidant Power, *Journal of Chromatography A*, **2003**, 1012, pp 31-38.