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Determining the Gallic Acid Concentrations in Red Wines using Reverse Phase HPLC with UV-Detection

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Abstract

Polyphenols are a form of antioxidants which consist of a three-membered flavan ring system that bind to metal cations inhibiting the cations' ability of binding to DNA and producing free radicals. Such free radicals are known to cause neurodegenerative and cardiovascular diseases. Wine, resulting from fermenting grapes with their skin components, contains polyphenols. Due to the pigment within the skin of the grape used in fermentation, red wine has higher amounts of these polyphenols and the amount and type of polyphenols within red wine has been shown to decrease the amount of cholesterol in blood. However, it has been shown that iron-deficiency anemia may arise, have worsened symptoms, or may be prolonged when red wine is consumed that contains the polyphenol gallic acid. This study utilizes refurbished High Performance Liquid Chromatographs (HPLC) with Ultraviolet (UV) detection to measure the gallic acid polyphenol in red wines because red wines decrease risks of neurodegenerative and cardiovascular diseases yet gallic acid increases the risk of chronic anemia. The research involved creating a method of extracting and analyzing gallic acid concentrations in three different types of red wines known for having high resveratrol concentrations. The study found there was variance among different brands of the same type of red wine and variance among red wine types.

1. Introduction

Wine is a beverage resulting from fermentation of yeast and lactic bacterial cells from the juice of a mash grape.[1] It begins its history almost around the same period as civilized humans began theirs. 6000 years before the Common Era, wine had many names including the Greek term, *methu*, or fermented honey water, which in English translates to *mead*. After many years, the more commonly known version of wine appeared and began its wide spread dispersion from western Iran to eastern France; by the year 50 AD becoming a standard choice of beverage due to its dysentery-bacteria free state.[2]

One contribution of its popularity is due to the hardiness of the wine source: the grapevine. The grapevine is a highly productive plant which can adapt to a wide range of soils and climates. Since the grapevine has adapted to a multitude of climates and soils, many different types of wines have been produced.[2] From the grapevine, the grapes are plucked, mashed, and fermented with sulfur dioxide, to produce wine. Since the main component is grapes, wine includes sugars, such as glucose and fructose; acids, like tartaric and malic acid; and pigment and aroma molecules.[1] The diversification of the grapevine births different varieties including red and white/green grapes. These grapes lead to the commonly-consumed categories of red and white wines. These grapes, other than having various fermentation processes, lead to a variety of wines available which contain different molecular compounds.

Wine, resulting from fermenting grapes with their skin components, contains a pigment molecule called anthocyanin derived from the grape skins. Anthocyanin is a compound within a large class of molecules called polyphenols.[3] Polyphenols are a most abundant form of antioxidants which consist of a three-membered flavan ring system.[4] Some of the most common polyphenols are tannin, resveratrol, and anthocyanin.[5] The health effects of

polyphenols are due to binding to metal cations. When metal cations, for example Mg^{2+}, Fe^{2+}, Fe^{3+}, bond to the DNA-phosphate back bone and react with hydrogen peroxide released during this oxidative stress period, they produce free radicals. The radicals released cause cell death when the metal-bound DNA creates an oxidative stressed-environment; the metals, for example iron, that are bound to the proteins, such as hemoglobin, are then unbound and released. The matriculation of unbound iron leads to neurodegenerative diseases, Alzheimer's and Parkinson's, as well as cardiovascular disease and increases the risk of cancer. The deprotonated oxygen atoms on polyphenols chelate to metal cations and prevent bonding of the metal cation to the DNA strand.[3]

Red wine has higher amounts of polyphenols from the skin pigments used in fermentation.[4] About 431 mg of polyphenols per serving were reported in red wines whereas 92 mg of polyphenols per serving were reported for white wines.[4] The amount and type of polyphenols within red wine is the main contributor for the French Paradox where the French cuisine consists of a high fat diet and moderate consumption of Pinot Noir, Cabernet Sauvignon, Merlot and Cabernet Franc red wines.[6,7] This paradox has been reported to be due to the polyphenol resveratrol, which is a molecule that becomes oxidized via the chelation of Cu^{2+}.[7] Resveratrol oxidation prevents the oxidation of low density lipoprotein (LDL) and thus lowers the risk of cardiovascular disease and heart attack.[4] However, red wine consumption has been shown to worsen symptoms of anemia due to polyphenols binding to iron within the blood stream and the gastrointestinal tract.[8]

More specifically, iron-deficiency anemia occurs when there is a lower amount of red blood cells in the blood due to the decrease of iron absorbed in the blood stream and gastrointestinal tracts. A protein called transferrin transports the iron from digested food in the digestive tract and transports iron to the liver where iron is stored as ferritin. When the body requires new red blood cells after about 120 days, iron is transported from the liver to the red bone marrow where new red blood cells are produced. With an increased intake of polyphenols, iron binds to the polyphenols instead of transferrin and, therefore, cannot be stored in the liver. Since iron is required for the production of new blood cells, reduced iron absorption results in lack of red blood cells in the blood stream causing the anemia.[9]

Having chronic anemia can lead to an increased risk of cardiovascular disease due to increased heart-pumping activity.[8] A polyphenol that has been studied having higher affinity for iron binding, and therefore have a direct relationship to iron-induced anemia is gallic acid.[10] Because of the multitude of positive health effects with the polyphenolic content of red wine, it would be beneficial to find a wine that has the optimum polyphenolic content that decreases risks of neurodegenerative and cardiovascular diseases while having a lower amount of gallic acid to decrease the risk of iron induced anemia. This would require a method of extracting and analyzing different polyphenolic compounds.

Other studies have already evaluated the antioxidant capabilities of polyphenols in red wines. One study measured the antioxidant capacity and polyphenolic content in 1 to 3 year old vintage wines including Vranec, Merlot, and Cabernet Sauvignon to see if there was a difference in time and the antioxidant capacity. The study used High Performance Liquid Chromatography (HPLC) to measure the polyphenol anthocyanin for each wine with a reverse phase column and a photodiode array detector. It was found that the age difference between the wines had no affect on the antioxidant capacity and that the concentration of anthocyanin in each wine did not vary significantly between wines of the same hue.[11]

Another study used Gas Chromatography Mass Spectroscopy (GC-MS) to determine the amount of free polyphenolic compounds in red wines. The study aimed to determine a method for mass-analyzing hundreds of wine samples with clear GC-MS peaks. To create clear peaks with rapid sample preparation, Bond Elut Priority PolLutant (PPL) cartridges were used to extract the compounds and the polyphenols were subjected to acid hydrolysis and injected into the GC-MS. It was found that this process improved accuracy and precision for determining the amount of polyphenols in wine samples.[12] The extraction of these polyphenols might be used to enhance the peaks derived in a HPLC study for measuring compounds that elute at the same retention time.

Past HPLC studies were conducted in order to determine the polyphenolic content of red wines. HPLC with diode array detection was used to determine if wines that were darker in color had more or less polyphenols than wines with a lighter color. The wine was diluted with HCl and using a solution of acetonitrile, water, and formic acid as a mobile phase it was determined that the darker red wines had more polyphenolic content.[13]

Other standard ways of measuring polyphenols use HPLC with UV detection. The Prevail column company used two solutions: 25mM KH_2PO_4, acidified to a pH of 2.5 using HCl, and acetonitrile, at a flow rate of 1 mL per minute and detection at 280nm. The compounds elute from the reverse phase C18 column within 20 minutes.[14]

The objective of the research was to quantify gallic acid in red wines known to contain high resveratrol concentrations (Pinot Noir, Cabernet Sauvignon, and Merlot) using HPLC with UV detection to determine the gallic acid concentration and variance among wines. Results enabled determination of a wine type that has a lower concentration of gallic acid that will reduce the risk of iron-deficiency anemia while still having an optimum

polyphenolic content for cardiovascular health. During the summer of 2013, a fully functional HPLC system was reconstructed by combining and repairing about 30 non-functioning HPLC components including pumps, UV detectors, and autosamplers donated from hospitals and other chemistry institutions. This instrument was used for all analyses.

2. Experimental Methods

2.1 Instrument Refurbishment

The summer of 2013 was spent repairing and reconstructing the HPLC devices. HPLC instrument refurbishment entailed disassembling and reassembling HPLC devices from multiple non-functioning devices to create two fully functioning systems. All of the HPLC devices declared dysfunctional beyond repair were properly disposed. Once the instrument towers were assembled and connected to a signal integrator to quantify detector output, each of the reverse phase columns was tested. Instrument function and acceptability of a C-18 Phenomenox column was confirmed with separation and UV detection of pure standards of caffeine in deionized water at 20, 40, 60, 70, 80, and 100 ppm (µg/mL) within acceptable error.

2.2 Gallic Acid Extraction and Analysis

The mobile phase gradient used to analyze gallic acid with HPLC[15] was created from two Liter solutions of 2.5% acetic acid in deionized water for Solution A and 99.8% methanol in deionized water for Solution B. The gradient consisted of starting with 100% Solution A and 0% Solution B, slowly increasing the concentration of Solution B over 30 minutes, and ending with 100% Solution B. A 2500 ppm stock solution of gallic acid was prepared from solid gallic acid diluted with deionized water, that was further diluted to create gallic acid standards of 25, 50, 75, and 100 ppm. A 10 ppm gallic acid standard was made by diluting the 100 ppm standard. The standards were kept in the refrigerator until they were analyzed with HPLC. The standards were injected three times at 15µL volumes at a detector wavelength of 280 nm with the mobile phase gradient at a flow rate of 1 mL/min.

To determine the gallic acid concentrations in wine and to analyze the wine samples with HPLC, wine samples were collected a week before analysis and included Pinot Noir, Cabernet Sauvignon, and Merlot. Of each wine type, three different brands were collected. For Pinot Noir, Red Diamond (2011), Acaria (2012), and Picton Bay South Island (2013) were collected. For Cabernet Sauvignon, only cool climates area wines were collected which included B&G (2013), Bogle Vineyards (2012), and Hob Nob Vineyards (2010). For the Merlot wine samples, Wente (2011), Wild Meadows (2011), and Genesis (2012) were collected. Each sample was not allowed to breathe and was immediately poured into clean 25 mL vials to the top to allow for minimal exposure to the air. The samples were then kept sealed in plastic baggies in a refrigerator until HPLC analysis.

The Pinot Noir-Red Diamond sample was the first to undergo HPLC analysis for gallic acid. The sample was filtered using 0.45µm nylon syringe filters and injected into the instrument at 15µL volume, the UV detector was set at 280 nm with the mobile phase gradient. Undiluted acetonitrile was then introduced as the mobile phase after one hour of sample analysis.

To decrease the residence time wine samples on the column, an extraction method for gallic acid was developed and used for all wine samples. Several attempts of extracting gallic acid at a high percentage were made using bases sodium bicarbonate, ammonium hydroxide and calcium hydroxide for deprotanation and organic solvents ethyl acetate, hexanes, petroleum ether. In the optimum extraction procedure, each wine sample was filtered using a nylon 0.45 µm nylon syringe filter and 8mL of the sample was added to about 0.170g of sodium bicarbonate. The solution was washed three times with 8mL of petroleum ether and 20 drops of 1 M HCl was added to the aqueous layer. Each solution was then diluted to 10 mL and injected three times at 15µL sample injections, with a 280 nm detector wavelength using the mobile phase gradient. The five gallic acid standards also underwent HPLC analysis using the same instrument settings.

3. Results and Discussion

3.1 Instrumentation Refurbishment

An HPLC instrument is composed of separate devices working in unison, including a pump, injection valve, separation column, detector and display. All of the devices, when connected together, create a functioning system. The mobile phase is pumped into the injection valve in the autosampler by the pump. The sample is injected into the mobile phase flow by the autosampler and the mobile phase-sample flows from the injection valve through the column and into the flowcell of the UV detector. The detector measures the absorbance by the sample and sends a signal to the Shimadzu signal integrator which plots the chromatogram with absorbance with respect to time and those peaks are integrated for the peak areas. These peak areas are directly proportional to concentration and allow for concentrations to be determined and quantified. Two Perkin Elmer Series 200 HPLC devices were refurbished to full function, with the exception of the blue tower's injection value which had a clog within the stator. This clog caused the pump to exceed it maximum pressure limit; a new stator piece is required.

The repaired instrument was used for caffeine analysis in a teaching lab environment. A calibration was constructed and shown in Figure 1. When the instrument response, or peak area, is plotted against caffeine concentration, a linear regression is produced which allows for an unknown concentration of caffeine to be determined. The linear regression has an r-squared value of 0.9979. Thus, column separation and UV detection are reliable and should produce reliable results gallic acid analysis.

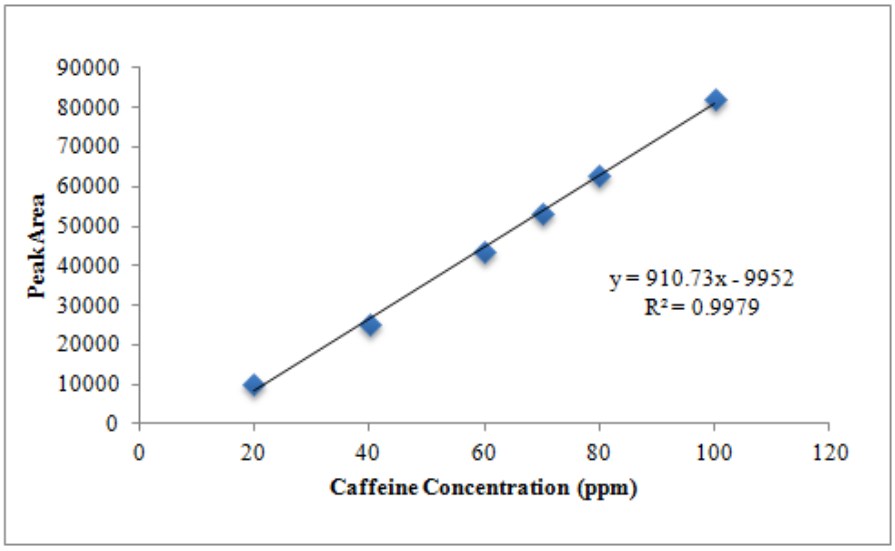

Figure 1. Caffeine calibration curve of instrument response peak area vs. caffeine concentration in ppm.

3.2 Gallic Acid Analysis

In order to quantify the gallic acid within the wine samples, gallic acid standards were prepared and analyzed using HPLC. The standards were injected three times into the instrument. Table 1 shows the peak areas for each standard. When the instrument response, or peak area, is plotted against concentration, a linear regression is made which can then be used to quantify gallic acid concentrations of the samples. Figure 2 plots this linear correlation where the x axis is the concentration of the gallic acid standard in ppm and the the y axis is the instrument response (peak area). The Limit of Detection (LOD) and Limit of Quantitation (LOQ) obtained from the calibration curve are 2.4 and 7.3 ppm, respectively, which indicates that the HPLC will be able to detect low concentrations of gallic acid using the prepared standards.

Table 1. Instrument response peak areas for gallic acid standards

Gallic Acid Standard	Peak Area (arbitrary units)
10 ppm	2648
25 ppm	6605
50 ppm	13257
75 ppm	20705
100 ppm	27329

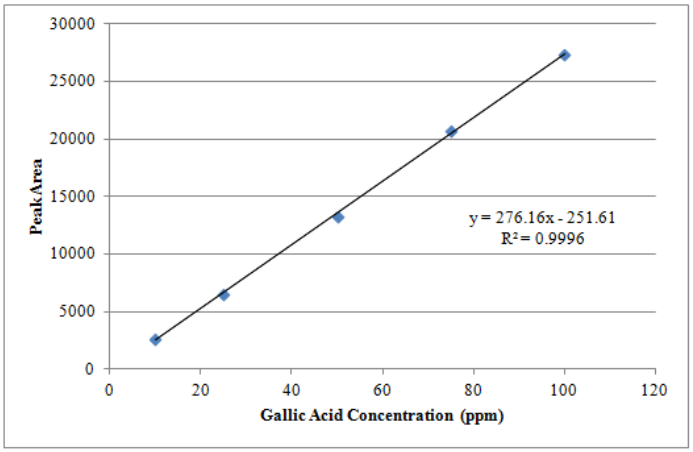

Figure 2. Gallic acid calibration curve of instrument response peak area vs. gallic acid concentration in ppm.

To determine the gallic acid in wine samples, the wine was filtered and the injected into the HPLC instrument. In the first injection of the Pinot Noir-Red Diamond wine, gallic acid eluted within the first 5 minutes. However, the other components of the wine sample demonstrated a high residence time, approximately five hours, in the column. Acetonitrile was used to decrease the residence time after the completion of the mobile phase gradient, but the residence time was still far too long to efficiently measure gallic acid.

To decrease residence time of the sample in the column, an extraction method was constructed in order to remove the more nonpolar compounds within the wine sample that were causing the high residence time, while still enabling accurate quantification of gallic acid. The extraction method employed a base to deprotonate the carboxylic acid and hydroxyl groups of the gallic acid, causing high gallic acid solubility in the basic aqueous layer, and an organic solvent to extract the less polar compounds. It was found that bases that had a pH higher than 10 reacted with the gallic acid itself and there were no peaks on the chromatogram. The only base that had a pH lower than 10 was sodium bicarbonate which proved weak enough to only react with the phenolic hydroxyl groups and the carboxylic acid of the gallic acid and not react with the hydrogens on the benzene ring. Petroleum ether was used as the organic layer because it is nonpolar enough to not extract the gallic acid from the aqueous layer, but polar enough to extract the undesired compounds causing the long residence time. It was also found that if the organic solvent was too polar, then all of the detectable gallic acid would be extracted into the organic layer. But if the organic solvent was too nonpolar, it would not extract moderately polar compounds causing high residence time.

To prove the efficiency of the extraction method and the amount of gallic acid retained post-extraction, the extraction method was performed on a 75 ppm gallic acid standard three times. Table 2 shows the peak area of each injection for the three extractions with the mean peak area and standard deviation. The variance of each peak area is 5.7% which is a low relative error in the extraction method. When compared to the peak area of the 75 ppm standard without the extraction, 49.5 ± 2.7% of the gallic acid was extracted from the 75 ppm standard. The 49% gallic acid extraction accounts for both the 8 to 10 mL dilution of the standard and the efficiency of the liquid-liquid extraction method. Taking the 8 to 10 mL dilution into consideration and adjusting the peak area to volume ratio for before and after extraction, the extraction method extracted 62.3% of the gallic acid from the original standard with high reproducibility.

Table 2. Instrument response of extracted 75 ppm gallic acid solution.

Peak Area (arbitrary units) of extracted 75 ppm standard	Mean Peak Area ± Standard Deviation (arbitrary units)
9415 10401 10422	10079 ± 575.4

The percentage of the extraction can then be used to determine the concentration of gallic acid in the three wine samples. Since petroleum ether had optimum polar/nonpolar properties, the residence time decreased from 5 hours to approximately 30 minutes with the same mobile phase gradient.

Using the linear regression, shown in Figure 2, and the 49% extraction efficiency, the pre-extraction gallic acid concentrations was determined for each of the red wine brands. Table 3 reports each brand of Pinot Noir, Cabernet Sauvignon, and Merlot with its peak areas, concentrations with the propagated error, and the mean concentration of each brand with its standard deviation.

In order to determine the variance of the red wines, a t-test was used to determine whether the each brand would be statistically the same or different. It was found that, with both 95 and 99% confidence, that the Acaria and Red Diamond red wines had statistically the same amount of gallic acid. It was found that Acaria and Red Diamond, when each individually compared using a t-test to Picton South Island, were each statistically different in gallic acid concentrations. This determines that there is variance among Pinot Noir wines.

For both the Cabernet Sauvignon and Merlot brand variance, when compared using the t-test, it found that the Cabernet Sauvignon wine brands were all statistically different from one another. The Merlot brands were also statistically different from each other when compared at a 99% confidence level. This determines that there is variance among brands of both Merlot and Cabernet Sauvignon.

When each wine variety is compared to one another, it was found that Cabernet Sauvignon had the largest variation in gallic acid concentration ranging from 25 to 78 ppm. The Pinot Noir and Merlot wines had a smaller gallic acid concentration range with a difference of approximately 20 ppm between the highest brand average and the lowest brand average. When the wine brands are averaged together, Merlot has the lowest gallic acid concentration average of 38 ppm, Cabernet Sauvignon has the median concentration average of 49 ppm, and Pinot Noir has the highest averaged gallic concentration of 60 ppm. The averages indicated that there is variance among the red wine types. However, when no longer comparing the types of wine but the brands of wines, Cabernet Sauvignon's large gallic acid concentration range indicated that some Cabernet Sauvignon brands could statistically have the same amount of gallic acid as Pinot Noir or Merlot brands. The Cabernet Sauvignon B&G brand and the Pinot Noir Picton Bay South Island brand both have gallic acid concentrations that are statistically the same. The Merlot Genesis brand and the Cabernet Sauvignon Bogle brand are statistically the same and the Cabernet Sauvignon Hob Nob brand is also statically the same as the Merlot Wild Meadows brand.

The statistical analysis of the different brands of wine is met with confidence because the standard deviation of each wine is low, between 0.6 and 6 ppm, which demonstrates that there is low variance among samples injections for each brand of wine. The method also contributes to the confidence of the t-test due to the low propagated error which was between 2 and 5 ppm. Since the propagated error was small, the devised method including the extraction was shown as being a sufficient manner in which to collect, extract, and inject gallic acid when it is present in a wine sample.

Table 3. Each wine type with their three brands' peak areas, concentrations with propagated error, and mean with the standard deviation.

Type	Brand	Peak Area (arbitrary units)	ppm ± Propagated Error	Mean ± SD
Pinot Noir	Picton Bay South Island (2013)	10282 9286 9670	77 ± 5 ppm 70 ± 5 ppm 72 ± 5 ppm	73 ± 4 ppm
	Acaria (2012)	7085 6003 7490	54 ± 4 ppm 46 ± 4 ppm 57 ± 4 ppm	52 ± 6 ppm
	Red Diamond (2011)	7164 7400 8263	54 ± 4 ppm 56 ± 4 ppm 62 ± 4 ppm	57 ± 4 ppm
Merlot	Wente (2011)	5171 5121 5549	40 ± 3 ppm 39 ± 3 ppm 42 ± 3 ppm	40 ± 2 ppm
	Wild Meadows (2011)	6616 6491 6660	50 ± 4 ppm 49 ± 4 ppm 50 ± 4 ppm	50.0 ± 0.6 ppm
	Genesis (2012)	3680 3225 3355	29 ± 3 ppm 25 ± 3 ppm 26 ± 3 ppm	27 ± 1 ppm
Cabernet Sauvignon	B&G (2013)	10919 10340 10335	82 ± 5 ppm 77 ± 5 ppm 77 ± 5 ppm	78 ± 2 ppm
	Hob Nob (2010)	6256 6250 5259	48 ± 4 ppm 47 ± 4 ppm 40 ± 3 ppm	45 ± 3 ppm
	Bogle Vineyards (2012)	3135 3316 3314	25 ± 3 ppm 26 ± 3 ppm 26 ± 3 ppm	26.6 ± 0.8 ppm

4. Conclusion

A fully functional HPLC system was reconstructed using donated devices and then used to evaluate gallic acid concentrations in Pinot Noir, Merlot and Cabernet Sauvignon red wines which are known for having high concentrations of resveratrol. The gallic acid was first extracted from wine samples and then analyzed by HPLC with UV detection. It was found that Pinot Noir had the highest-average gallic acid concentration of 61 ± 11 ppm while Merlot had the lowest averaged concentration of 39 ± 12 ppm. It was also determined that Cabernet Sauvignon had the largest gallic acid range in concentration with a difference of 26 to 78 ppm. As for the comparison of brands within a type of wine, it was found that there was variance among the different brands of red wine types. Pinot Noir and Merlot had variance in gallic acid concentrations, however the wide range of gallic acid concentrations in Cabernet Sauvignon shows invariance in gallic acid concentrations when compared to certain brands of Merlot and Pinot Noir wines.

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