ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

DETERMINATION OF TOTAL POLYPHENOLS IN ETHIOPIAN RED WINES BY REACTION WITH 4-AMINOANTIPYRINE AND PHOTOMETRIC FLOW INJECTION ANALYSIS

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DETERMINATION OF TOTAL POLYPHENOLS IN ETHIOPIAN RED WINES BY REACTION WITH 4-AMINOANTIPYRINE AND PHOTOMETRIC FLOW INJECTION ANALYSIS

A Graduate project to be submitted to the office of research and graduate program of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in chemistry.

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Declaration

I the Undersigned confirm the results reported in this work were obtained by research carried out by me under the supervision of my Advisor in the Faculty of Science, Department of Chemistry, Addis Ababa University in the academic year 2006-2007. No part of this work shall be published in scientific journals or reported in the media or presented at a conference without the knowledge and consent of my advisor, who is the principal scientist responsible for any publication. Furthermore if the work is published the institutional address given should be that of the Chemistry Department, AAU.

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Place and date of submission: School of Graduate Studies
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List of abbreviations and acronyms

4-AAP	4-aminoantipyrine
^{o}C	Degree Celsius
CHD	Coronary Heart Disease
FAO	Food and Agriculture Organization
FIA	Flow Injection Analysis
FI	Flow Injection
GA	Gallic Acid
GAE	Gallic Acid Equivalent
GC-MS	Gas Chromatography–Mass Spectroscopy
HCF	Hexacyanoferrate(III)
HDLC	High Density Lipoprotein Cholesterol
HPLC	High Performance Liquid Chromatography
HPLC-DAD	High Performance Liquid Chromatography–Diode Array Detector
IV	Injection Valve
LDL	Low Density Lipoprotein
LLE	Liquid-Liquid Extraction
mgL^{-1}(mg/L)	milligram per liter
g/L(gL^{-1})	gram per liter
PP	Peristaltic Pump
PPO	Polyphenol Oxidase
PVC	Polyvinyl Chloride
RC	Reaction Coil
RSD	Relative Standard Deviation
SD	Standard Deviation
SPE	Solid Phase Extraction
w/w	weight by weight ratio
v/v	volume by volume ratio

Abstract

A simple flow injection spectrophotometric method for the determination of total polyphenols in red wines is reported. The method is based on the oxidative coupling of polyphenols with 4-aminantipyrine in alkaline medium to yield a colored product with maximum absorption at 470 nm. After optimization by univariant approach, the linear range was established between 9.4 to 451.5 mgL^{-1}. The proposed system was applied for the determination of total polyphenols in Ethiopian red wines using gallic acid as the standard and the results are given as gallic acid equivalent (GAE) in mgL^{-1} with RSD < 1%. The method enabled analysis of approximately 43 samples h^{-1}. The selectivity of the method was evaluated using compounds that are normally present in wine such as sugars, tartaric acid, ascorbic acid, methanol, ethanol, ammonium sulfate, and potassium chloride and it was found to be fairly selective (relative error < 4.2%). The method was validated through recovery test and the result was in the acceptable region 90.5% - 107.3%.

1. Introduction

1.1 Wine history as part of the human culture

Wine has been part of the human social, economic, and religious culture since 6000 years [1]. The Neolithic period, from about 8500-4000 B.C., is the first time in human prehistory when the necessary preconditions came together for the momentous innovation of viniculture [2]. The exact date when the cultivation of grapevines and winemaking began has been highly debated due to the lack of historical evidence. Some historians suggest that the first domestication came around 8000 B.C. in Asia Minor and Transcaucasia, but this suggestion has been thrown into doubt for evolutionary reasons. More evidence supports the theory that winemaking began at some point between 6000 and 4000 B.C. in the mountainous region between the Black and Caspian Sea, near the borders of the modern states of Turkey, Iraq, and Iran [3].

The earliest chemical evidence so far dating 5400-5000 B.C., was discovered in Iran. A pottery jar from a Neolithic village in Iran's northern Zagros Mountains that contained wine was discovered [1, 4, 5]. This is two thousand years earlier than the evidence from the earliest civilization of the near east.

Some investigators place the origin of wine making, or at least its development, in the southern Caucasus. It is also thought that the domestication of the wine grape (*Vitis Vinifera*) initially occurred with in this area. It is there that the natural distribution of *Vitis Vinifera* most closely approaches that of the western viniculture suggesting spread from the former to the latter [1].

There are frequent references to wine in the Old Testament. Wine was also used by early Minoan, Greek, and Etruscan civilizations. Centuries later the role of wine for sacramental use in Christian churches helped maintain the industry after the fall of the Roman Empire [6].

In progress of spreading west, towards Europe, grape vine reached, coming from the East Mediterranean area, first Greece and later on Italy, France and Central Europe. Almost simultaneously with the westward extension, the cultivation of vine spread towards East over Iran, Pakistan and India so that it reached China at the end of the second century B.C. [3].

The exact time of wine introduction to Ethiopia is not yet known. However traditions extend it back to the days when the Christian missionaries and traders settled [7].

Today, *Vitis vinifera* has reached all continents but is successfully cultivated only in temperate climate regions with warm and dry summers and relatively mild winters with sufficient rain [8]. Natural factors make wine from a particular region unique. Known in the wine industry as *terroir,* these factors include local climate (temperature, rainfall, and sunlight), location of grapevines (altitude and slope), and soil (structure, composition, and water drainage). In general, a grapevine produces the best fruit when the moderate climate provides much sunshine and cool nights without frost, and the soil is well drained. Grapevines grow best in sandy, chalky, or rocky soils but generally require long, dry, warm-to-hot summers and cool winters for best development [9].

In Ethiopia grapes from varieties originally imported from Armenia, Italy and Sicily are grown on the hills of Dukam, Gouder, Abadir, Zway and Debre Zeit [7].

Wine is the fermented juice of grapes. Only one species of grape, *Vitis vinifera*, is used for nearly all the wine made in the world. From this species as many as 4000 varieties of grape have been developed. These varieties differ from each other, though sometimes only slightly, in size, color, shape, juice composition, ripening time, and resistance to disease. Of the 4000 or more varieties, only about a dozen are commonly used for wine making around the world. The chief varieties are: Riesling, Chardonnay, Cabernet Sauvignon, Pinot Noir, Gewurztraminer, Sauvignon Blanc, and Muscat [6].

The high sugar content of most *V. vinifera* varieties, when ripe, is the main reason for their use in wine production. Grape varieties of *V. vinifera* have a great range of composition. Skin pigment colors vary from greenish yellow to russet (chocolate color), pink, red, reddish violet, or blue-black. The color of red wines comes from the skin, not the juice. The juice is normally colorless, though some varieties have a pink to red color. Juice flavors vary from mild to strong [6].

1.2 Wine production and consumption in the world

Annual global wine production averaged nearly 280 million hectoliters (approximately 28 million metric tons) during the 1998 to 2002 period. During that same period, 67 countries reported commercial wine production to the United Nations Food and Agriculture Organization (FAO) [9]. Taking into account the long-term trends and the changes in trends that occurred in the middle of the 1990's, it can be estimated that the world production of wines should approach 300 million hectoliters on the 2010 timescale [10].

The world production of wine remains dominated by Europe, representing nearly 67.1% of output in 2005. America follows with 19% of the world production in 2005, the other continents representing between 3.6 and 5.4% of the world production of wine [10].

Global trends in the wine industry show that Europeans are the leading consumers of wine, and accounted in 2003 for more than 68% of the 228 million hectoliters of wine consumed every year worldwide. America, overall, ranks second in terms of world consumption, with a quota of just over 20%, while Asia ranks a distant third, with 6.6%. Luxembourg, France and Italy rank top for per capita consumption. The French and Italians drink about 50 liters of wine per person/year. These two countries are also the leading world consumption markets, followed by the USA, where per capita consumption, however, is about 7.5 liters [11].

Taking into account the long-term trends and the changes in trends and recent rises, it can be estimated that by 2010 global wine consumption could range between 239 and 255 million hectoliters, that is 9 to 10 million hectoliters more than today [10].

In Ethiopia the only wine industry Awash Winery, is producing 9.5 million liters of wine annually. The factory makes seventeen different sorts of wines. A major portion of the factories production goes for domestic market. Export performance, on the other hand, is steadily growing and is gaining international recognition [7].

1.3 Wine making process (Vinification)

The process of wine making undergoes a serious of unit operations leading from the crushing of grapes to the bottling of wines. The common sequences used in the production of white and red wines are shown in figures 1 and 2 respectively. The basic procedures in commercial wine making process are;

1.3.1 Harvesting

This is the most critical stage of the process. The grapes must be harvested when the sugar, acid, phenol and aroma compounds are optimized for the style of wine desired [12].

1.3.2 De-stemming and crushing

Immediately after harvest, extraneous material such as stems, leaves, and grape stalks are removed. This minimizes the excessive uptake of phenols and lipids from vine components. The grapes go into a stemmer/crusher, which both separates the individual grapes (berries) from the stems and leaves, and breaks open the skins to allow the juice to run free. This juice is then called the "free run." The grapes are then placed in a press and depending on the type of wine to be made; various degrees of pressure can be exerted on the grape skins/pulp to extract more juice. Generally, white wines are made from juice without the skins, while red wines are fermented with skins and seeds included. With red grape varieties low in phenol content, extraction of stem phenols is valuable. Stem phenols generally produce more astringent and bitter tastes than phenols released by the seeds and skins [1, 12, 13].

1.3.3 Maceration

Following crushing of the grapes the rapture and release of enzymes from grape cells facilitate the liberation and solubilization of compounds bound in cells of the skin, flesh, and seeds. Maceration is almost always employed in the initial phase of red wine fermentation. Limited maceration is used for white wines with slight juice oxidation before fermentation. In red wine production, the style of the wine can be altered dramatically by the duration and conditions of maceration. Macerations less than 24 hours commonly produce rose wines. Macerations of 3-5 days generally produce wines for early consumption with good coloration and low tannins. Wines for long aging are usually macerated on the seeds and skins from 7 to as long as 21 days. This may enhance color stability and aging potential [1].

1.3.4 Pressing

The juice extraction process depends on the type of wines to be used, but always involves squeezing the berries. There are three types of press: horizontal, pneumatic (membrane), and continuous screw press. Winemakers can influence the character of wine by the choice of the press. The degree of fining and blending of the various press fractions allows winemakers to adjust the final character of the wine [1, 12].

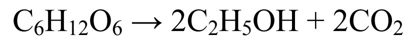
1.3.5 Thermovinification

Thermovinification is one technique of improving the color of red wine. It involves the heating of intact or crushed grapes between 50°C and 80°C. The temperature varies depending on the varieties. Thermovinification is used primarily to produce wines designed for early consumption [1].

1.3.6 Fermentation

Saccharomyces cerevisiae is the principal agent in wine fermentation, converting the simple sugars of grapes to carbon dioxide and alcohol. Once the juices of the fruit have been exuded, an ideal breeding ground with the right mix of water and nutrients is created for *S. cerevisiae* to begin and sustain fermentation to produce an alcoholic beverage [5].

The amount of alcohol produced is controlled by the amount of yeast and the duration of fermentation (*i.e.* a larger quantity of yeast and longer fermentation produces more alcohol). The net overall reaction can be summarized as: [14]



$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

This reaction occurs through many intermediary biochemical steps. The process is carried out under a blanket of carbon dioxide as in the presence of oxygen the phenols are oxidized and the sugar and ethanol are converted to carbon dioxide and water [12].

1.3.7 Purification

Proteins and tannin that are suspended in colloidal form in the wine are precipitated out with substances such as gelatin or adsorbed to the surface of substances such as bentonite. This process is called fining. The wine is often also clarified in a process called raking. This is the drawing off of the wine from the lees (sediments formed). Wine is also cold stabilized (left at 0 to -3 ^{o}C for 10-14 days) to crystallize any potassium bi-tartarate. The wine is continuously raked off this precipitate, such that by the end of ageing process all it needs is simple filtration before bottling and sale [12].

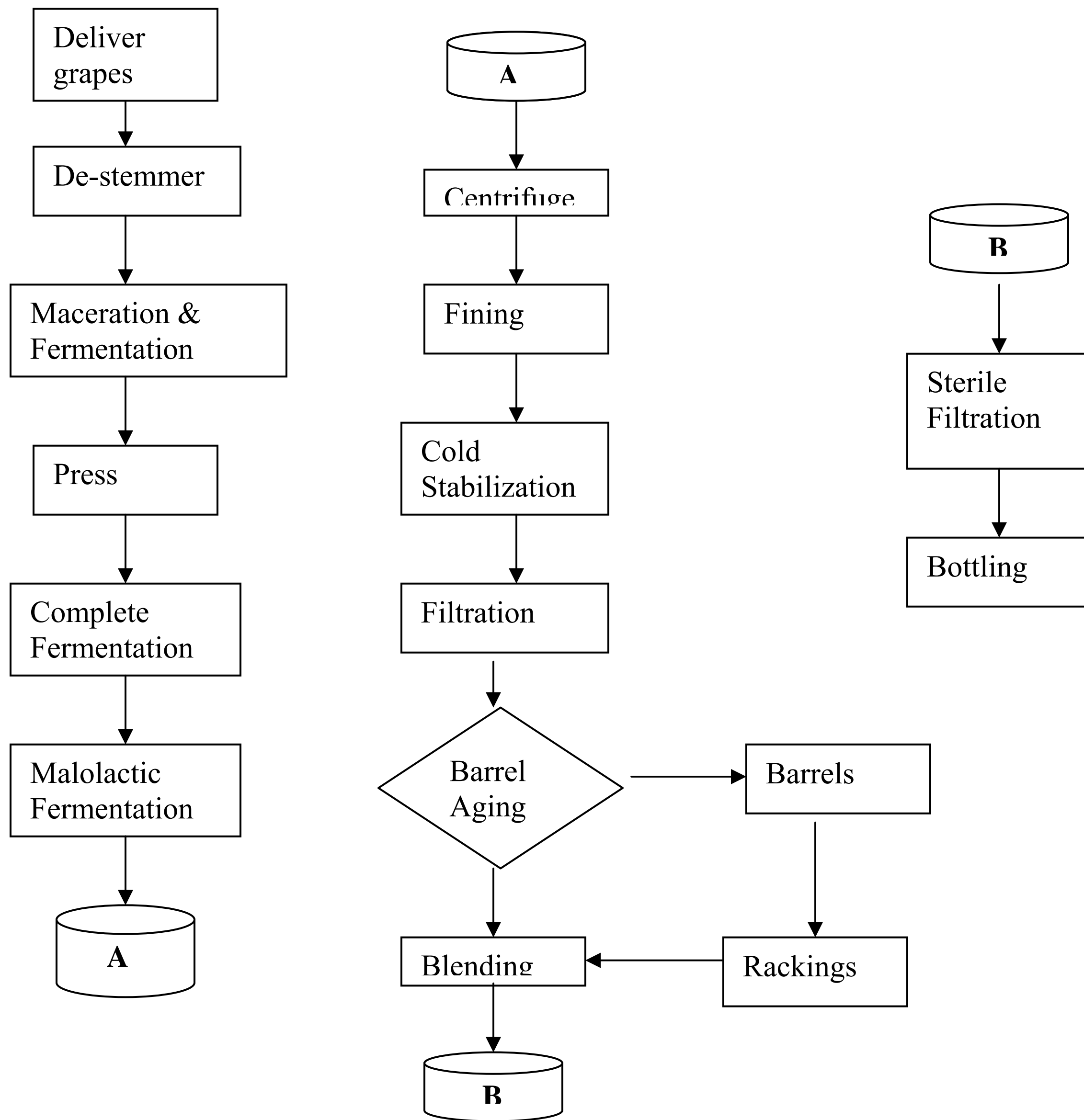


Figure 1 General structure of unit operations for the production of white wine [1].

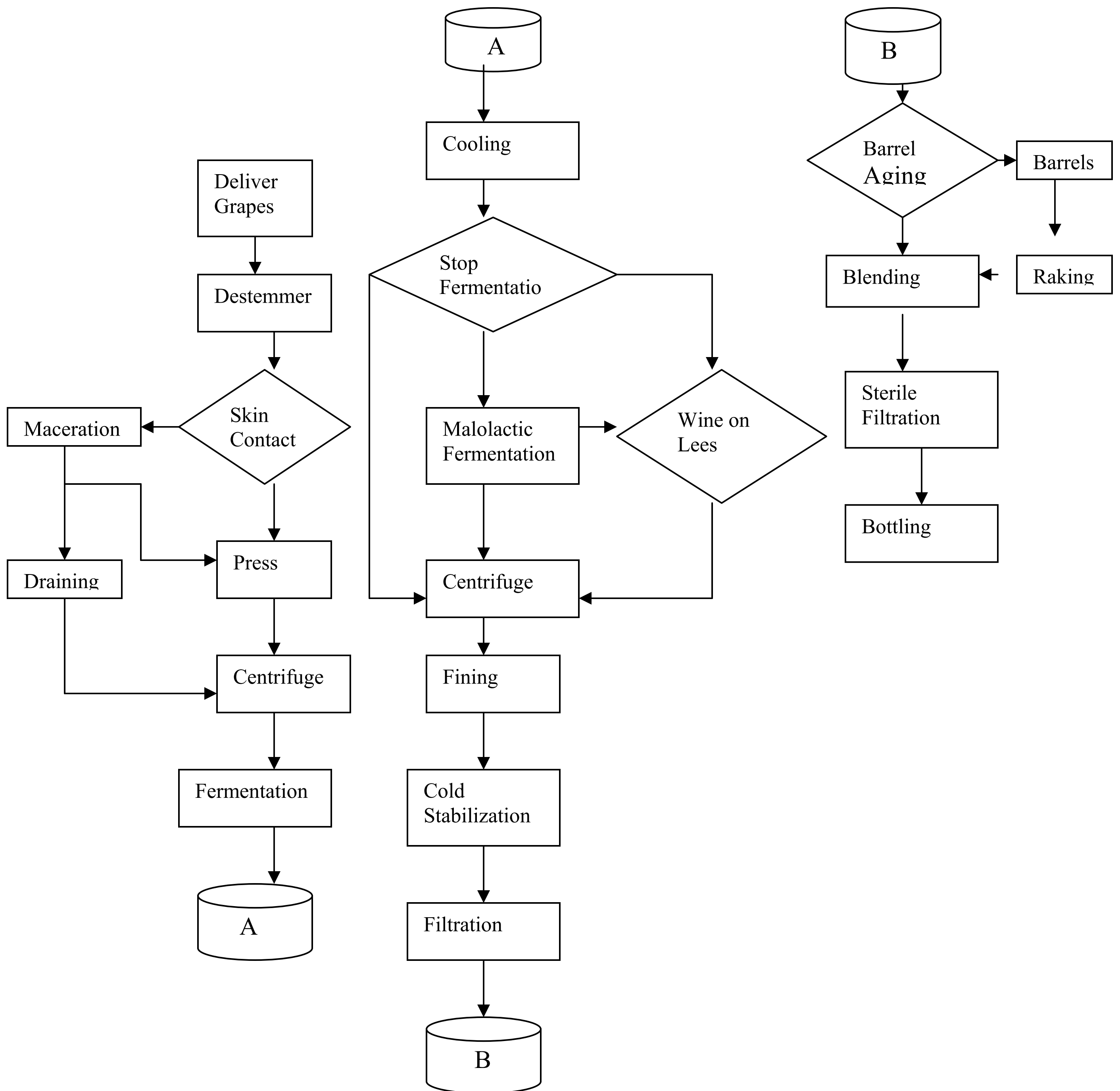


Figure 2 General structure of unit operations for the production of red wine [1].

1.4 Chemical composition of wines

More than 500 compounds have been recognized in wine thus far, of which 160 are esters. The concentrations of the majority range between 10^{-1} and 10^{-6} mg/L. At these

levels the individual compounds play very little or no role in the human organoleptic perception, but collectively they may be very significant [15].

Water is the predominant chemical constituent of wine and wine grapes. It is an essential component in many of the chemical reactions involved in grape growth and juice fermentation and in wine aging. Compounds insoluble or only slightly soluble in water rarely play a significant role in wine [1].

Principal sugars are glucose and fructose, and they occur in roughly equal proportions at maturity of grapes. The primary wine yeast, *Saccharomycets cerevisae*, derives most of its metabolic energy from glucose and fructose and has limited ability to ferment other substances. In addition to being absolutely essential for fermentation and production of ethanol, sugars are metabolized to higher alcohols, fatty acid esters, and aldehydes, which give different wines their individual aromatic character [16].

The most important and abundant alcohol in wine is ethanol [17]. Under standard fermentation conditions, ethanol can accumulate to 14-15%, but generally ethanol concentration in wine range between 10-13%. The primary factors controlling ethanol production are sugars, temperature, and yeast strain. Methanol is a minor constituent of wine and has no direct sensory effect. It is predominantly generated from the enzymatic breakdown of pectins [1].

In wine, acids are divided into two categories: volatile and fixed. The first refers to acids that can be readily removed by distillation, whereas the latter refers to the carboxylic acids. The most common volatile acid in wine is acetic acid. Quantitatively, carboxylic acids such as tartaric, malic, lactic, succinic, oxalic, fumaric, and citric acids control the pH of wine [18].

1.4.1 Phenolics

The term 'phenolics' refers to a large group of compounds containing at least one phenol group (i.e. containing at least one hydroxylated benzene ring). These compounds

contribute to the astringency / bitterness of the grapes and wine and are responsible for most of the color [12].

The phenolic composition of wine depends not only on the phenolic composition of the grapes but also on the winemaking conditions that influence their extraction. Winemaking processes such as maceration temperature and cold soaking influence the extraction of phenolics. There are two classes of phenols in grapes and wine: Flavonoids and Non-Flavonoids [19].

1.4.1.1 Non-Flavonoids

Cinnamic and benzoic acid derivatives are the main non-flavonoids present in grapes and wine (Figure 3). In grapes these non-flavonoids are usually bound to glucose and esters. Non-flavonoid concentrations are in the order of 10-20 mg/L in white wines and 100-200 mg/L in red wines [20].

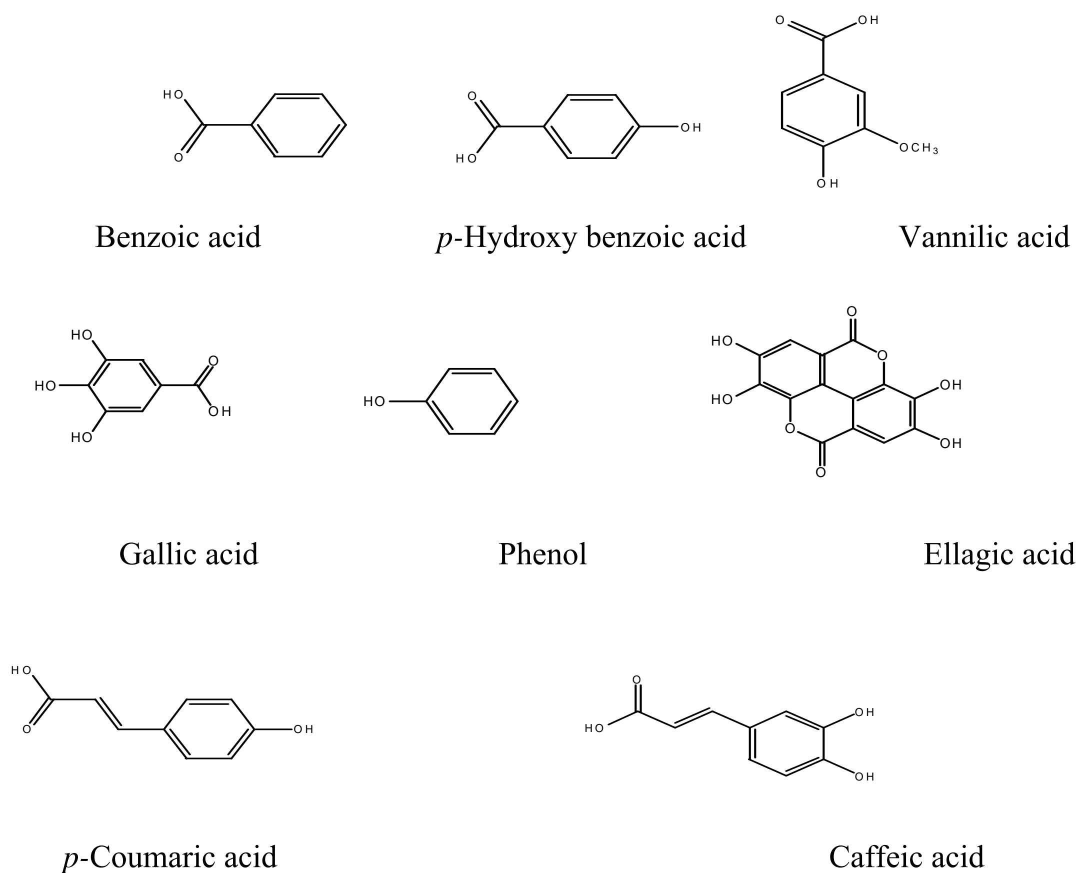


Figure 3. Examples of different non-flavonoids from wine [19].

1.4.1.2 Flavonoids

Flavonoids are more complex than non-flavonoids and consist mainly of two benzene cycles bonded by oxygenated heterocycle (fig. 4). Flavonols, flavan-3-ols, flavan-3,4-diols and anthocyanins are major classifications of flavonoids depending on substituents on the flavonoid skeleton [19].

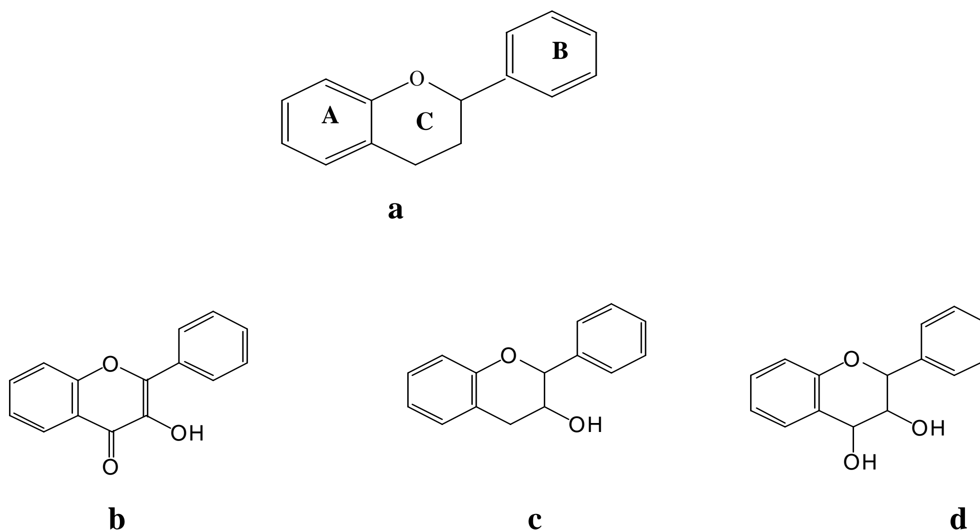


Figure 4. a) Flavonoid skeleton with standard letter system, b). Flavonol, c). Flavan-3-ol, d). Flavan-3,4-diol [1, 19].

The difference between red and white wines is due to the different types of phenolics in the two beverages. The simple phenolics, hydroxycinnamates and hydroxybenzoates occur in the flesh of the berry and so occur in both red and white wines. The other more complex phenolics, known collectively as flavanoids, occur in the skin, seeds and stems and so occur mostly in red wines. The procyanidins are also known as condensed (or non-hydrolysable) tannins, and it is these that give wine most of its astringency. A further group of tannins, the hydrolysable tannins, are found in wine that has spent time in oak barrels. These tannins are also astringent, and are complex esters of glucose and gallic acid. Anthocyanins are the commonest source of color not only in grapes but also in all flowering plants. Their color depends on the number of hydroxyl groups on the molecule and can range from orange through red to purple [12].

Table 1. Composition of wine excluding phenolic acids and polyphenols [17].

Component	**Concentration (g/100mL)**
Water	80-90
Carbohydrates	
Glucose	0.05-0.1
Fructose	0.05-0.1
Pentoses	0.08-0.2
Inositol	0.03-0.05
Fucose	0.0005
Alcohols	
Ethyl	8-15
Other	0.3-0.19
Glycerol	0.30-1.40
Aldehyde	0.001-0.05
Organic acid	0.3-1.10
Nitrogenous compounds	0.01-0.09
Mineral compounds	0.15-0.40

Table 2 Gross phenolic composition for a typical red wine [1].

Phenolic class	Amount as GAE (mg/L)
Non-flvonoids, total	240-500
Cinnamates, derivative	150
Low volatility benzene, derivative	60
Tyrosol	15
Volatile phenols	15
Hydrolizable tannins	0-260
Macromolecular complexes	
Protein-Tannin	10
Flavonoids total	705
Catechins	150
Flavonols	10
Anthocyanins	20
Soluble tannins, derivative	450
Other flavonoids, derivative	75?
Total phenols	**955-1215**

1.5 Red wine consumption and health effect

Epidemiological evidence from many studies, involving hundreds of thousands of human subjects of both sexes, overwhelmingly supports the notion that light-to-moderate alcohol consumption is associated with a reduction in overall mortality, due primarily to a reduced risk of coronary heart disease (CHD) [1, 17, 21-23].

Further, an inverse relation has been found between alcohol intake and platelet aggregation, this has been shown to be even stronger for wine [24]. It was reported that the type of alcoholic beverage in addition to its quantity, could be important for the protective effect on mortality. In that study, for 3 to 5 drinks per day, cardiovascular mortality was reduced by 47% in the wine drinkers, but the other causes were also 50% lower. An important issue was that the other causes were not reduced in the drinkers of beer and spirits [25].

The most popular incidence, 'the French Paradox' is a solid evidence for the negative correlation between wine consumption and CHD. The French, in spite of high saturated fat consumption, comparable plasma cholesterol and similar risk factors showed considerably lower incidence of death from CHD than Americans did. This incompatibility of a diet rich in fatty foods with a decreased risk of heart disease bears the name 'the French Paradox' [1, 22].

Scientific evidences make it clear that wine polyphenols act through various mechanisms in addition to chemical antioxidant action to effectively reduce disease-provoking processes and provide protection that is separate from that afforded by moderate ethanol intake [17].

Different mechanisms were proposed in how the polyphenols tackle the associated cardiovascular health problems. Most of the results show polyphenols are able to increase high density lipoprotein cholesterol (HDLC), which is known for its negative health factor for CHD. The other mechanism is it inhibits platelet aggregation and blood clotting. Investigators demonstrated both *in vivo* and *in vitro*, a reduced aggregation of platelets altered eicosanoid metabolism leading to a reduction in thromboxane synthesis coupled with increased synthesis of vasodilatory protacyclin. Other mechanisms may reduce the likelihood of atherosclerosis, such as inhibition of low density lipoprotein (LDL) oxidation, free radical scavenging and modulation of eicosanoid metabolism [1].

Saint-Cricq de Gaulejac *et.al.* [26] reported that red wine has great antioxidant potential, due to the phenolic compounds (tannins and anthocyanins) which are present in sufficient quantities to ensure optimal free radical scavenging activity of the compounds and even combined action between them leading to a synergic effect of these polyphenols.

When wine extracts containing oligomeric procyanidins were diluted 1,000 times, their activity in inhibiting the oxidation of isolated human LDL far exceeded that of the vitamin C and E [27].

Other health benefits associated with moderate consumption of red wine are, decreased risk of human malignancies, including colon, breast, lung, larynx, pancreas, bladder, stomach, esophageal, and oral cancer [28]. It also protect against gastritis induced by *Helicobacter pylori* infection [29, 30], protection of skin damage from sunlight [31], and reduced risk of demantia [32].

Some findings however identified some negative effects of red wine. Risk of dependence on alcoholism and a commonly associated crime takes the premier. The other problem is dental erosion up on prolonged consumption. Red wine affected the oesophageal and gastric potential, indicative of gastric distress, more than coca-cola and whisky. The observed changes were thought to be due to changes in Cl^- secretion and/or due to a damaging effect of the additives of the beverages. Most red wines release serotonin from blood platelet that causes migraine headache [23].

1.6 Polyphenol determination methods from red wines

An overall knowledge of the phenol content of wine, expressed as a numerical value, provides winemakers with enough information about polyphenol concentration. This makes possible to classify wines according to their phenol content and measure the results of a winemaking operation [33].

Many techniques were developed to study the polyphenol content of wines and different grape extracts. Some of these methods are used to determine total polyphenol content, but some are restricted to a specific group or class of polyphenols.

The method of choice for the separation, isolation, purification, and identification of polyphenols is chromatography. Chromatographic techniques will not only separate individual polyphenols but also give both quantitative and qualitative results. Paper chromatography and thin-layer chromatography were the earliest techniques developed, and they are still used today [27].

Recent publications have shown high performance chromatography (HPLC) to be the method of choice in the analysis of phenolic compounds in wines [34].

Due to their high concentrations in red wines, anthocyanins can be determined simply by injection of samples into an HPLC-DAD instrument. On the other hand, phenolic acids, catechins, procyanidins, and flavonols usually require a concentration and fractionation pretreatment [35].

Most frequently used pretreatment techniques are mainly liquid-liquid extraction (LLE) and solid phase extraction (SPE) [34, 35]. This method of analysis however, requires high analysis cost, and labor. Moreover, the pretreatment methods can result in errors in estimating the time.

Goldberg *et.al* [36] reported the use of gas chromatography-mass spectroscopic (GC-MS) method for the determination of phenolic constituents of red-wines. The method was also preceded by sample pretreatment and derivatization. The drawbacks associated with the technique are, it requires longer analysis time (about 26 minutes per sample) high cost and errors at the time of sample pretreatment.

A number of spectrophotometric methods have been developed to quantitate the levels of phenolic compounds in wine and plant material. The Folin-Ciocalteu assay is the widely

used method that can measure total phenolics in food products. It is oxidation-reduction reaction, where the phenolate ion is oxidized while phosphotungstic-phosphomolybdic compounds are reduced to a blue molybdenum-tungsten complex that is then measured at 760 nm. The phenol content is determined by using the standard gallic acid as a reference [19, 27, 37].

Although adapted by the organization international du Vin, and still widely used, this method suffers from interference from several compounds, including ascorbic acid, reducing sugars, iron (II), nucleic acid, amino acids, and proteins. Another disadvantage is that the color evolves with time, making it very difficult to obtain accurate results [37]. Again it leads to overestimation of phenols because all hydroxyl groups are oxidized [19].

Electrochemical detection based on immobilized polyphenol oxidase (PPO) enzyme was reported [38-40]. Polyphenol oxidases are copper containing oxygenases catalyzing the *o*-diphenols to the corresponding *o*-quinones. These enzymes are often called tyrosinases, phenol oxidases and polyphenol oxidases [39]. The biosensing approach has advantages of high specificity, high sensitivity and rapid detection mechanism. However, the difficulty in immobilizing the enzyme, relatively short lifetime, susceptibility to temperature change and pH makes it less useful. Moreover, the enzyme is incompatible with benzoates that occur naturally in wines [38].

An alternative strategy used for quantitative analysis in recent decades is flow-injection analysis (FIA), which has several advantageous features, including versatility, simplicity, and low cost [37].

Flow injection (FI) methods (commonly designated collectively as flow injection analysis, FIA), have proved to possess a high potential in almost every analytical field as they offer a simple means for automating a great variety of determinations [41].

The applicability of the method for wine analysis was reported for tartaric acid [18], reducing sugars [42], total poyphenols [37, 43], and total catechins and procyanidins [33]. Total polyphenols from wine were determined based on different chemical background. The Folin-Ciocalteau method was adapted to FIA, however due to the mentioned reasons above it is less applicable [37].

The present project employs the basic principle that phenols undergo oxidative coupling with 4-aminoantipyrine in alkaline medium to produce a colored complex [44].

Sample red wine solutions react with 4-aminoantipyrine at pH 9 in the presence of potassium hexacyanoferrate (III) and form a deep red tea colored complex. The absorbance of the reaction product is monitored at 470 nm. The height of FIA peak is proportional to the total polyphenol and the result is expressed as gallic acid equivalent.

2. Objectives

2.1 General objective

The objective of this project is to determine the total polyphenol content of Ethiopian red wines by using flow injection analysis.

2.2. Specific objectives

- To develop a flow injection analysis procedure for total polyphenol determination by a spectrophotometric detection.
- To measure the total polyphenol content of Ethiopian red wines.
- To measure the interference potential of some selected species in wine samples.
- To find optimum reaction parameters to determine polyphenols from wine in flow injection mode.

3 Experimental

3.1 Apparatus

The flow injection (FIA) manifold (figure 5) comprised a Gilson Minipuls 3 peristaltic pump (France) fitted with PVC tubing and a six-port Rheodyne 7725(i) sample injection valve with 20 μL sample loop (USA). The detection system was an LKB 2151 variable wavelength detector comprising a 10 mm flow through cell (KNAUER GmbH, Germany).

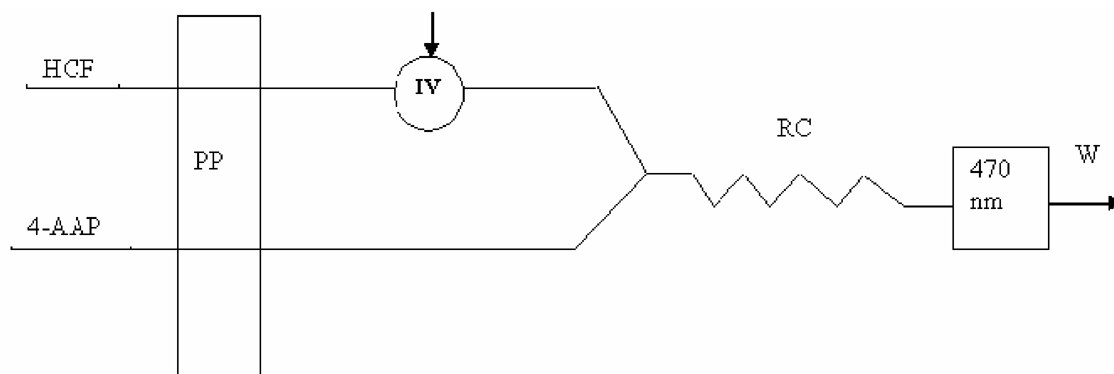


Figure 5 The flow injection manifold. HCF, Hexacyanoferrate; 4-AAP, 4-aminoantipyrine; PP, Peristaltic pump; IV, injection valve; RC, Reaction Coil; D, detector and W, waste.

Spectrophotometric readings under batch conditions were made by Beckman DU-65 spectrophotometer (USA) and polystyrene cuvetts (dispolab Kartell) with an optical path length of 10 mm.

The pH of the buffer solutions was determined with pH-301 pH/ion meter (HANNA instruments, Portugal) calibrated with standard pH buffers at 4.01, 7.01, and 10.01. All the mass measurements were taken by LARK (LA 114) analytical balance.

3.2 Reagents and Solutions

All the chemicals employed for the project were of analytical grade, and deionized water (conductivity < 1.5 $\mu S\ cm^{-1}$) was used.

Ascorbic acid (BDH, England), 4-aminoantipyrine (4-AAP), boric acid (Hopkins & Williams, England), potassium hexacyanoferrate (III) (HCF) (Merck, Germany), potassium chloride (BDH, England), gallic acid monohydrate (Riedel-de Haen), ammonium sulfate (Sisco Reasearch Laboratories, India), tartaric acid (Mallinckrodt, New York), sodium hydroxide (BDH, England), glucose(Sisco Reasearch Laboratories, India), sucrose (Sisco Reasearch Laboratories, India), fructose (Sisco Reasearch Laboratories, India), ethanol (J.T. Baker Chemicals, Phillipsburg), and methanol (ReAgent, England) were collected from the chemical store.

Buffer solutions of pH 9, 10, 11, and 12 were prepared by addition of 0.1 M aqueous boric acid solution to 0.1 M aqueous sodium hydroxide solution until the desired pH was obtained.

Gallic acid (GA) standard solutions were daily prepared in buffer by appropriate dilution of a stock solution. This last solution, usually 1881.4 mgL^{-1} of GA was prepared by accurate weighing of the corresponding solid and appropriate dilution with buffer.

4-AAP solution was prepared in 25 mL of deionized water to a final concentration of 4.065 gL^{-1}. The oxidizing agent HCF was usually prepared by dissolving 164.63 mg of the solid in a final volume of 50.0 mL of buffer. Dilute solutions of oxidizing agent and 4-AAP were obtained by appropriate dilution of stock solutions with buffer.

Interference from glucose, sucrose, fructose, tartaric acid, ascorbic acid, methanol, ammonium sulfate, and potassium chloride were evaluated for 188.14 mgL^{-1} GA solutions in buffer prepared with or without the interfering compound at 100% (w/w) with respect to GA.

3.3 Wine samples

The red wine samples were from Awash winery, Ethiopia. The samples were identified by the brand names; Gouder, Axumite, and Dukam. Three products are known by the brand name Gouder, two for local consumption differ in size and closure (the bigger with wine stopper the small with simple bottle cork) and one for export standard. All the samples have the same alcoholic content of 11.5% (v/v). Each of the five samples was purchased from a local grocery and kept at room temperature till analysis.

3.4 Procedures

The proper wavelength for maximum absorbance was obtained from DU-65 Spectrophotometer under batch condition scanning from 400–700 nm. All the other optimizations were followed after fixing the wavelength at 470 nm in FIA mode.

To select the appropriate pH for the formation of the colored complex, standard GA solution of concentration 188.14 mgL^{-1} was prepared in buffer solutions of pH 9, 10, 11, and 12. All solutions of 4-AAP (406.5 mg L^{-1}) and HCF (1.646 gL^{-1}) were prepared in the respective buffer. Optimization of pH was done keeping the flow rate at 0.56 mL/min.

The optimum flow rate was selected by recording the absorbance peak height at different flow rates between 0.30 to 1.80 mL/Min.

The HCF concentration was evaluated in the concentration range of 329.25 mgL^{-1} to 5.1 gL^{-1}.

4-AAP was optimized in the concentration range of 101.6 mgL^{-1} to 2.032 g L^{-1}.

The optimum results obtained after univariant optimization were used for calibration curve using GA as a standard in the concentration range 9.4 mgL^{-1} to 451.5 mgL^{-1}. The GA standard solutions were prepared in buffer (pH 9) and injected through the injection

valve. The GA was made to react first with 1.317 gL^{-1} HCF and afterwards mixed with 4-AAP solution 406.5 mgL^{-1}. These reactant solutions were prepared in buffer of pH 9.

Under the experimental conditions selected for FIA, red wine samples were injected in to the manifold after 10-fold dilution with a buffer of pH 9. The results obtained for the real sample was employed for the method validity test by the spiking procedure with gallic acid standard solution.

3.5 Sample preparation

All the five samples were simply diluted with a buffer solution prior to analysis. The dilution factor was 10 times for each sample.

4. Results and Discussion

The variables that affect the determination of polyphenols were studied by univarient approach depending on their interdependence and the optimum values are employed to the next step. All the measurements were carried out in triplicate and the average value is used.

4.1 Batch trials

The proper wavelength for maximum absorbance was evaluated in the visible range. Both the oxidizing agent and 4-AAP were set as background and scanned in the range of 400-700 nm. The maximum absorbance was attained at 470 nm (Fig. 6). This wavelength value was directly applied to the flow injection (FIA) system.

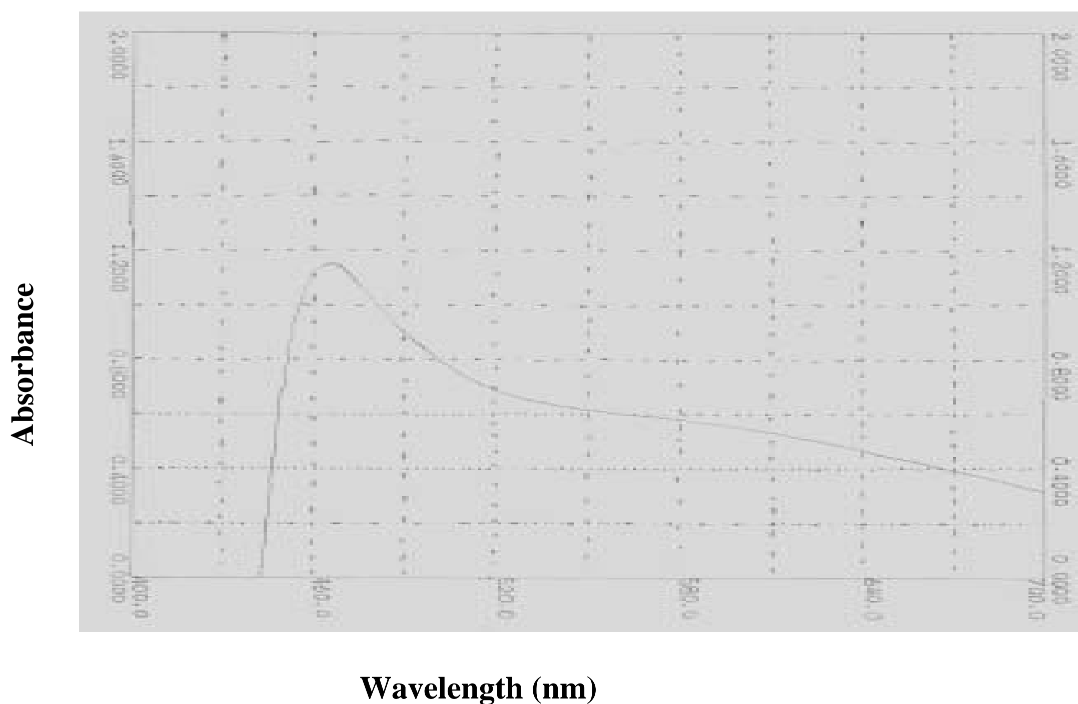


Figure 6 Visible spectrum obtained for the reaction product against HCF and 4-AAP background.

4.2 Optimization and characterization of the FIA method

4.2.1 Buffer pH optimization

The effect of pH was evaluated at pH 9, 10, 11, and 12; this range was selected because the reaction between phenols and hexacyanoferrate (III) (HCF) and 4-aminoantipyrine is usually better under alkaline solutions. Schoonen *et. al*. [37] reported that the maximum sensitivity for the oxidative coupling of GA with 4-aminoantipyrine (4-AAP) to be 11 under batch conditions (without checking for pH 9). In a FI mode pH 9 was found to be better (Fig. 7). The repeatability based on the standard deviation is almost comparable in all the pH values considered. Therefore, buffer pH 9 was used throughout the rest of the measurements.

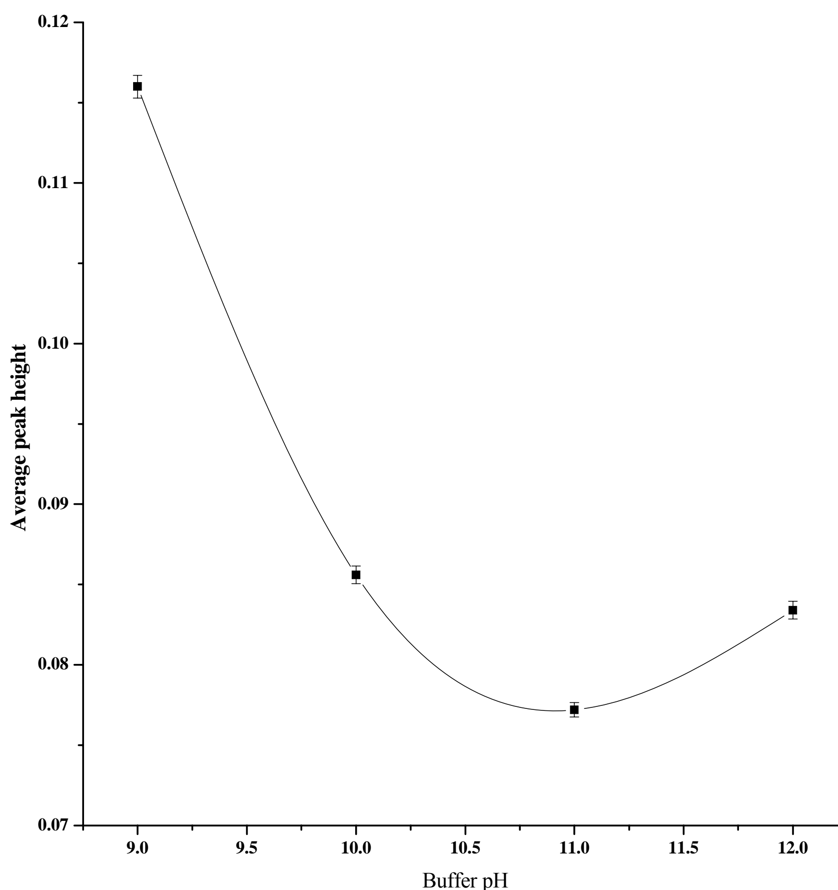


Figure 7 Effect of pH on the absorbance peak height at 4-AAP concentration 407 mgL^{1} HCF concentration 1.646 gL^{-1} GA concentration 188 mgL^{-1} at a flow rate of 0.56 mL/min.

4.2.2 Flow rate optimization

It is obvious that the response in any flow injection analysis is dependent on the flow rate. Therefore, its effect was on the absorbance peak height was investigated by changing the flow rate from 0.3-1.8 mL/min. Theoretically, one may expect higher signal at lower flow rate because of the longer residence time for the analyte in contact with the reagents and hence more color development. In this work, increase in flow rate led to a slight increase in signal (Fig. 8). That is due to the fast-fading of the developed color as observed in a batch experiment. The result observed in the flow rate optimization further substantiate it as higher peak heights were observed for higher flow rates. Though the higher flow rate results in a better sample throughput, there is no significant difference in peak height above a flow rate of 0.84 mL/min and thus this flow rate was chosen for subsequent measurements.

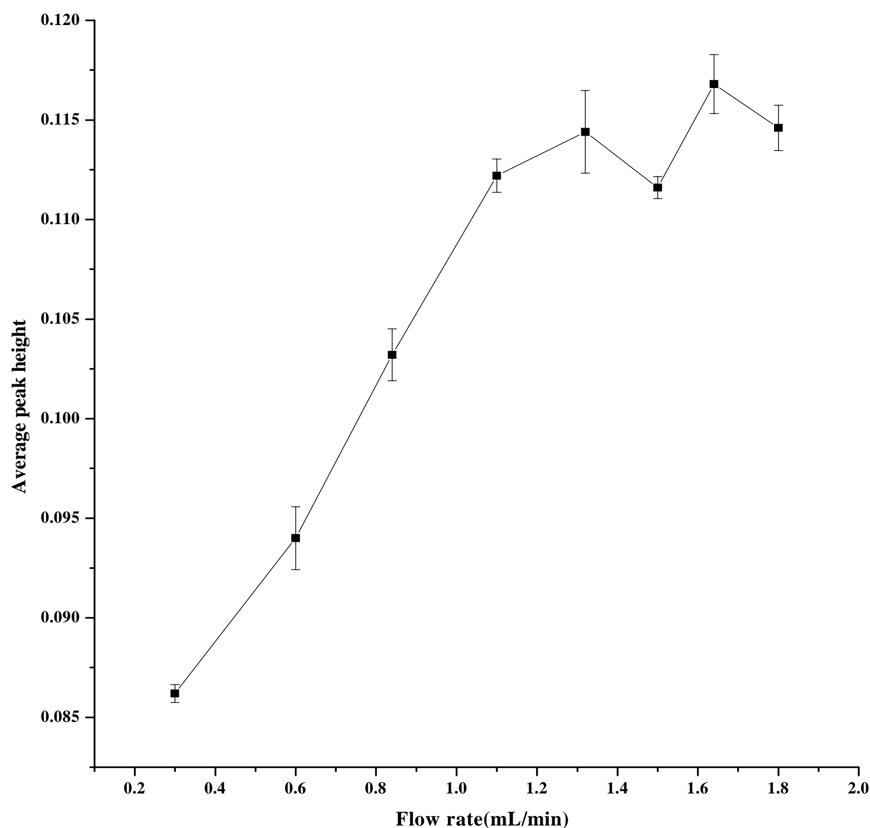


Figure 8 Effect of flow rate on the determination of polyphenols at concentrations of 4-AAP, HCF and GA 407 mgL^{-1}, 1.646 gL^{-1}, 188 mgL^{-1}, respectively at buffer pH of 9.

4.2.3 HCF concentration

The effect of the HCF concentration was evaluated by varying its concentration from 329.25 mgL^{-1} to 5.103 gL^{-1}. The absorbance peak height at first was increasing together with the concentration of HCF. After a maximum value the absorbance peak height decreases slowly as the concentration increases (Fig. 9).

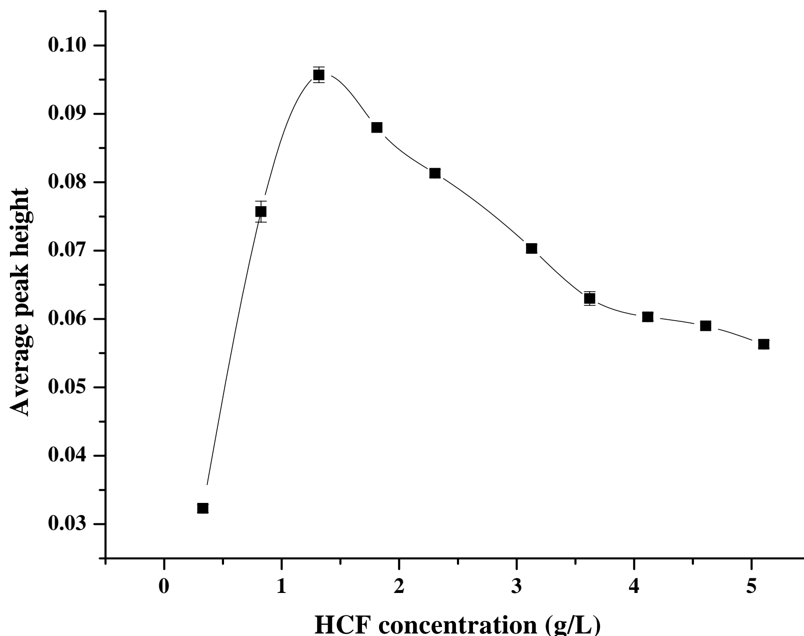


Figure 9 Effect of Hexacyanoferrate (HCF) on average peak height. Experimental Conditions were: Flow rate 0.84 mL/min, concentration of 4-AAP 407 mg/L, GA 188 mg/L, and pH 9.

The optimum concentration of HCF chosen was 1.317 g/L.

4.2.4 Concentration of 4-aminoantipyrine

The effect of 4-aminoantipyrine was studied from 102 mgL^{-1} to 2.033 gL^{-1} (Fig.10). The peak height slightly increases with increasing concentration till the climax at about 304.88 mgL^{-1}. Above this concentration the average peak height starts to decline. Therefore the climax value was taken for subsequent measurement.

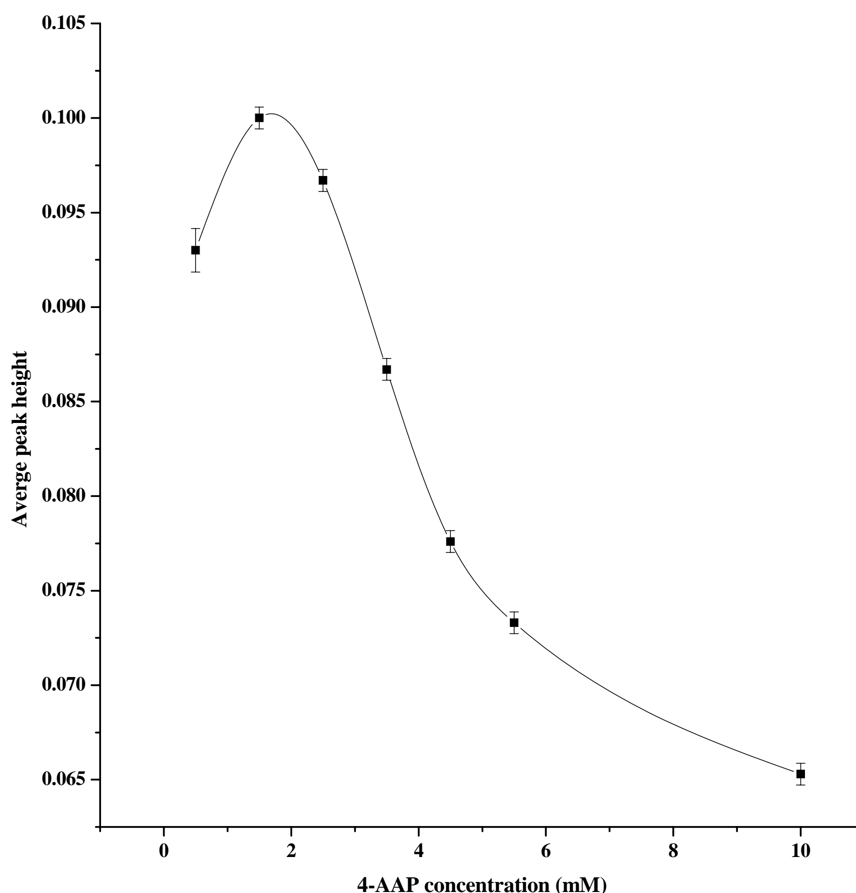


Figure 10 Effect of concentration of 4-AAP on average peak height. Experimental conditions were: flow rate 0.84 mL/min, concentrations of GA 188 mg/L, HCF 1.317 g/L, buffer pH 9.

4.3 Calibration curve plotted

Calibration curve was run using standard solutions of gallic acid employing all the optimized experimental conditions. Each point in the curve was obtained as the average of a triplicate injection. Linear response was found in the concentration range 9.4 mgL^{-1} to 451.5 mgL^{-1} (Fig.11). The linearity parameters are appreciable with R value of 0.996.

Linear Equation

$$\mathbf{A = 6.13734 \times 10^{-4}C + 0.00534}$$

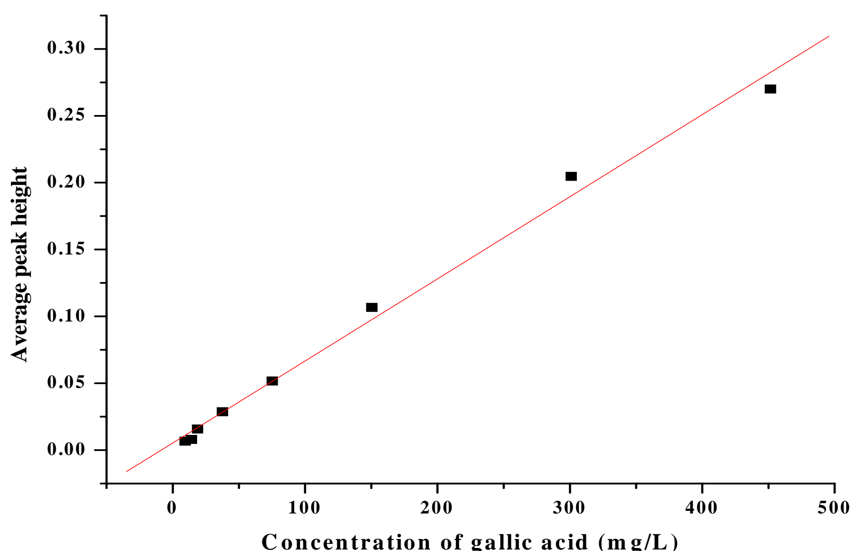


Figure 11. Calibration curve for Gallic acid. Experimental conditions were: flow rate 0.84 mL/min, concentrations of 4-AAP 305 mg/L, HCF 1.317 g/L, and buffer pH 9.

The calibration curve reveals that the results are precise from the invisibility of the error signals in all of the data points.

The detection limit calculated as three times the standard deviation of the peak height at the lowest concentration of gallic acid measured adjusted to concentration units was 2.82 mg/L.

The relative standard deviation value (RSD) for ten consecutive measurements at 18.8 mg/L of gallic acid was 3.3%.

4. 4 Analysis of red wine samples

Polyphenols were determined spectrophotometrically in red wines after calibrating the FIA system under the optimized conditions. Before analysis every sample was homogenized and diluted ten fold to fit the calibration curve. Results obtained for the five Ethiopian red wines from Awash Winery are listed in Table 3 as averages of a triplicate measurement.

Table 3 Total polyphenol content of Ethiopian red wines expressed as gallic acid equivalent (GAE mg/L)

Sample	Mean Polyphenol (GAE mg/L)[a]	% RSD
Gouder Local (Small)	662 ± 0	0
Gouder Local (Big)	776 ± 16	2
Gouder Export	1536 ± 9	0.6
Axumite	1097 ± 10	0.9
Dukam	1124 ± 9	0.8

[a] Value as mean ± SD (n = 3).

The results obtained for the samples are in good precision as apparent from the relative standard deviation (% RSD) that is less than 1 for all except Gouder Local (Big). Among the samples analyzed Gouder wine the export standard is with the maximum total polyphenol content. On the other hand the smaller local Gouder is with the least total polyphenol content. The significant difference in polyphenol content of the two local Gouder wines (smaller and bigger) might be due to the type of cork used to seal the bottle.

4. 5 Interference measurements

The interference potential of different compounds that are commonly encountered in wines was evaluated by recording the absorbance peak height both in the absence and presence of the interfering compound at 100 % (w/w) with respect to gallic acid. The results obtained are listed in Table 4. The investigation shows that methanol is relatively the interfering compound in the determination at the indicated concentration; however it is a minor constituent of wines (0.1–0.2 gL^{-1}) [1] hence its effect is negligible.

Fructose that is a natural grape sugar is the other interfering compound. But it is usually converted into alcohol at the course of wine fermentation and its availability is very small in fermented wines. The other compounds evaluated were found to be non-interferent for

the determination of gallic acid at their presence in a one to one ratio by mass with gallic acid.

Taking into account of the results obtained, it can be concluded that the method developed is fairly selective to the determination of polyphenols as gallic acid equivalent (GAE).

Table 4 Absorbance readings obtained from standard solutions of 188.14 mgL^{-1} Gallic acid prepared without or with a possible interfering compound at a level of 100 % (w/w) with gallic acid.

Interference Compound	Absorbance peak height		Relative error (%)
	Without interferent[a]	With interferent[a]	
Fructose	0.119 ± 0	0.122 ± 0	2.52
Glucose	0.119 ± 0	0.119 ± 0	0
Sucrose	0.119 ± 0	0.120 ± 0	0.84
Tartaric acid	0.119 ± 0	0.121 ± 0.001	1.68
Ascorbic acid	0.119 ± 0	0.120 ± 0.001	0.84
Methanol	0.119 ± 0	0.124 ± 0.001	4.20
Ethanol	0.119 ± 0	0.118 ± 0	- 0.84
Ammonium sulfate	0.119 ± 0	0.117 ± 0.001	- 1.68
Potassium chloride	0.119 ± 0	0.121 ± 0	1.68

[a] data are given as mean ± SD (n = 3).

4. 6 Method Validation

An attempt to compare the developed method with that recommended by the International Organization of wine (Folin-Ciocalteau method) was not successful due to unavailability of the reagents and therefore recovery was opted to validate the method.

All the five wine samples were diluted ten times and spiked with 3.22 mg of gallic acid in 25 ml volumetric flask.

Table 5 Recovery results obtained after spiking red wine samples with 3.22 mg GA in 25 ml volumetric flask.

Sample	**Measured GA(mg/25 ml)[a]**	**Spiked GA mg**	**Found GA (mg/25 ml)[a]**	**% Recovery[a]**
Gouder Local (Small)	1.66 ± 0	3.22	5.12 ± 0	107.3 ± 0
Gouder Local (Big)	1.94 ± 0.04	3.22	5.24 ± 0.04	102.4 ± 2.30
Gouder Export	3.84 ± 0.02	3.22	7.01 ± 0.02	98.2 ± 0.67
Axumite	2.74 ± 0.02	3.22	5.78 ± 0.02	94.3 ± 0.72
Dukam	2.81 ± 0.02	3.22	5.73 ± 0	90.5 ± 0.66

[a] data are given as mean ± SD (n = 3).

The results for the recovery are listed in Table 5 above. As clearly seen in the table, the recovery results are in the range of 90.5% to 107.3%, with a good precision. This shows the method is a very good alternative for the determination of total polyphenols from wines based on gallic acid equivalent. And as to the speed of analysis, it is possible to measure at least 43 samples an hour, which is fairly fast.

5. Conclusion

The FIA system proposed is simple, inexpensive, in terms of reagent consumption and equipments involved. And it enables analysis of approximately 43 samples h^{-1}. There was no interference of commonly available compounds in wine observed. Although the developed method was not validated in comparison with the recommended method, the recovery tests were satisfactory.

The total polyphenolic content of Ethiopian red wines was successfully determined by direct injection into the FI system after simple dilution with buffer. Therefore, the proposed method could be an alternative to routine determination of polyphenols in wines.

We expect this assay to be suitable for the analysis of total phenolic compounds in various types of wines and other chief sources of polyphenols, such as tea, potatoes, and apple with some sample pretreatment.

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