Addis Ababa University

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School of Chemical and Bioengineering

Extraction and Characterization of Antioxidants from Wine By-Product (Grape Pomaces)

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This is to certify that the thesis prepared by Tamirat Endale entitled: *Extraction and Characterization of Antioxidants from Wine By-Product (Grape Pomaces)* and submitted in partial fulfillment of the requirements for the degree of Master of Sciences (Chemical and Bio Engineering) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Advisor Adamu Zegeye (Associate Professor) _____ Signature _____ Date _____

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Acronyms

AAPH	2,2′-azobis(2-amidino-propane) dihydrochloride
A_{AR}	Antiradical capacity
ABTS	2,2′-Azinobis-(3-ethylbenzothiazole-6-sulphonate)
ANOVA	Analysis of Variance
AOAC	Association of American Analytical Chemists
CCD	Central Composite Design
CE	Catechin equivalent
CV	Coefficient of variation
DPPH	1, 1-diphenyl-1-picrylhydrazyl
DSC	Differential scanning calorimeter
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agricultural Organization
FCCCD	Face centered central composite design
FDA	Food and drug administration
GAE	Gallic acid equivalent
GPE	Grape pomace extract
HDL	High- density lipoprotein
HPLC	High Performance Liquid Chromatography
IA	Inhibition activity
LDL	Low-density lipoprotein
MhL	Millions of hectoliter
MT	Millions of tons
OIV	Organisation Internationale de la Vigne et du Vin
ORAC	Oxygen Radical Absorbance Capacity
PV	Peroxide value
RSA	Radical scavenging activity
RSM	Response surface methodology
RWGP	Red wine grape pomace
RWGPE	Red wine grape pomace extract
Std.Dev	Standard deviation
TAC	Total anthocyanin content
TBHQ	Tertiary butylhydroqunine
TE	Trolox equivalent
TEAC	Trolox equivalent Antioxidant Capacity
TFC	Total flavonoid content
TPC	Total phenol content
WGP	Wine grape pomace
WWGP	White wine grape pomace
WWGPE	White wine grape pomace extract

Abstract

Wine grape pomace (WGP), is a valuable by-product from the winemaking industries, recognized due to its relevant polyphenolic compounds. This study investigated the potentials of wine waste in Ethiopia from red wine grape pomaces(RWGP)and white wine grape pomaces (WWGP) (Vitisvinifera L.) for extraction of antioxidants. The effect of solvent ratio, extraction time and temperature on the yield of RWGP and WWGP extract was also studied. The experimental design was employed using the Design Expert 6.0.8, three-level-three factor Central Composite Design in triplicates at the center point in the optimization study, requiring 17 experiments. The results showed that minimum and maximum extract yields were 30.23% and 52.35% for RWGP, and 20.97% and 48.56% for WWGP, respectively. Solvent ratio, extraction time and Temperature had a significant ($p < 0.05$) effect on the extract yields. The statistical analysis indicates that, the linear terms of solvent ratio, extraction time and temperature, had positive effect on response yield. The quadratic terms (pure quadratic terms) negatively affected the extraction yield and only AB interaction for both RWGP extract and WWGP extract had negative influence. Characterization of antioxidants was carried through the evaluation of total phenolic content (TPC), total flavonoid (TF), total anthocyanin content and scavenging capacity against 1,1-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity performed using spectrophotometer. Additionally, food applications of the obtained extract to inhibit oil oxidation were carried out according to the Shaal Oven Test compared with butylated hydroxytoluene (BHT) by measuring per oxide value (PV). Results obtained under optimum conditions were: total phenolic content 15.70-56.72 mg GAE/g extract and 10.94-50.56 mg GAE/g extract; total flavonoid 49.65 mg CE/g extract and 18.56 mg CE/g extract; total anthocyanin content 530mg/L and 14.01mg/L and DDPH radical inhibition activity42.27%-98.50% and 39%-88.23% for RWGP extract and WWGP extract, respectively .The IC_{50} values for RWGP extract and WWGP extract were 1.24mg/ml and 1.49mg/ml and A_{AR} values were 0.80 and 0.67, respectively. The lower IC_{50} value the more potent antioxidant activity; and high value of A_{AR} refers to high antioxidant activity. Hence, RWGP extract has more potential in antioxidant activity than WWGP extract. Inhibition test of soybean oil oxidation showed that the extract was less than BHT, since the peroxide value was higher than of the control.

***Key words**: Red and White wine grape pomace; anthocyanins; antioxidant activity; flavonoid; phenolic compounds; yield*

Chapter One

Introduction

1.1 Background

Wine production is of great importance in agro-economic activities. The world grape production in 2012 exceeded 69 million tons and Europe was the largest producer of wine, with 66% of the total world production (Castañeda-Ovando *et al.,* 2009). The solid wastes generated by the wine industry represents between 25%–30% of the material used and it consists mainly of grape pomace (containing seeds, pulp, stem and skin) (Mendes *et al.,* 2013).

It is well known that high quantities of valuable compounds like dietary fiber, oils from the seeds, anthocyanins and phenolics compounds still remain within the grape pomace after processing (Prior and Schaich, 2005). The phenolic, such as resveratrol, have great potential due to their antioxidant capacity and health benefits against coronary diseases by the inhibition of LDL (low-density lipoproteins) and other chronic diseases, like cancer, diabetes and neurodegenerative disorders (El Gharras, 2009). In addition, from the economic point of view, the market of these compounds have been increased in the recent years by the increasing consumer demand for the use of more natural antioxidant compounds, achieving the value of US$30 billion, based on 2008 grape wine production data (Teixeira *et al.,* 2014). In this sense, the valorization and reuse of these wastes from the wine-making industry would have a significant environmental and economic impact, and this possibility has been studied by several authors (Chamorro *et al.,* 2012).

Antioxidants are important ingredients in food processing sectors. As their name implies, their role is to inhibit the development of oxidative rancidity in fat containing foods, particularly meat, dairy products, and fried foods. It was reported that antioxidants are ‘substances that when present in low concentrations with those of an oxidisable substrate, significantly retard oxidation of that substrate, (Gülçin, 2010). It is well known that plants are the richest sources of bioactive phytochemicals and antioxidant nutrients (El Gharras, 2009) .

It is now broadly accepted that certain classes of plant –based compounds such as dietary fiber, phenolic acids, flavonoids, vitamins, and antimicrobial agents and neuropharmacological agents play preventive role against the incidence of some common disease like cancer, cardiovascular and neurodegenerative disorders (Sáyago-Ayerdi *et al.*, 2009). In the modern food science era, the foods, which in the addition to imparting normal nutritive value also have disease protecting and physiological benefits, are known as" functional foods." The term, physiological functional foods, first appeared in Nature News in 1993 with the title "Japan explores the boundaries between food and medicine.

There are two categories of antioxidants; synthetic and natural. Synthetic antioxidants are generally compounds with phenolic structures of varying degrees of alkyl substitution which are created in laboratories, mainly for use in the preservation of foods. Conversely, natural antioxidants are obtained from plant or fungal sources; these include phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines) or carotenoids as well as ascorbic acid (Konczak, 2004).

Synthetic antioxidants such as Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT) and tertiary Butyl hydroquinone (TBHQ), have recently been found to cause carcinogenesis in rodents, and by analogy also possibly in man (Ignat *et al*., 2011), highlighting the benefits of antioxidants from natural sources such as from wine by products. For instance, Polyphenolic compound in grapes are known to lower oxidative stress, to modulate the inflammatory cascade, to reduce the oxidation of lipid and to induce protection against atherothrombotic episodes including myocardial ischemia and inhibition of platelet aggregation.

Most of these health effects have been ascribed to Polyphenolic compounds serving as reducing agents in many biological systems by donating hydrogen, quenching singlet oxygen, acting as chelators and by trapping free radicals. Moreover, these antioxidant activities help to limit oxidation of nucleic acids, proteins, lipids, which may initiate degenerative diseases such as cancer, heart disease, dermal disorders and aging.

1.2 Statement of the Problem

The importance of the antioxidants contained in foods is well appreciated for both preserving the foods themselves (especially fats, oil and fat containing food products), for preventing deterioration of other oxidisible goods, such as cosmetics, pharmaceuticals and plastics, and supplying essential antioxidants *in vivo* (Fontana *et al*., 2013). However, recently, consumers have rejected synthetic antioxidants because of their carcinogenicity, Since synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ), have restricted use in foods due to their toxicological effects on various species and suspected carcinogenic potential, the search of natural and safe antioxidants, especially of plant origin, has greatly increased in recent years. This, together with the fact that antioxidants are naturally present in many foods (oilseeds, nuts, cereals, legumes, vegetables, fruits, herbs, spices, teas and meat) and also the wine production increases in Ethiopia in recent years and using this byproducts for the extraction of antioxidant which decrease the cost of antioxidant and increasing the application of this for better preservation method that meet the interest of consumer. .

Additional in the last few years diminishing the environmental impact of industrial wastes has been a subject of increasing concern. Grapes production are one of the world's largest fruit crops, also in Ethiopia there are increasing production of wine and wine-making wastes are rich in phenols. These compounds considerably increase biochemical and chemical oxygen demands, while in solid residues used as fertilizers may inhibit germination. On the other hand, grapes, wine, grape seeds and skins extracts are reported to exert favorable effects on human health due to their phenolic content. The generation of waste by the wine industry has grown because part of the planted grapes are destined for wine production, which generates about 5% waste in the form of grape marc, in relation to produced grapes, and 4% as lees, in relation to the produced wine (El Gharras, 2009). One of the wine residues generated in greater amounts is the grape pomace. It consists mainly of skin and seed that are generated after concomitant maceration, alcoholic fermentation, and from grape pressing (in red wine or after pressing of the previously crushed grapes in the production of white wine).

1.3 Significance of the Study

Wine making by-products are a low-cost and rich source of valuable phenolic compounds with widely recognized health benefits. The recognized antioxidant properties of the phenolic compounds are based on their ability to scavenge free radicals; inhibiting the oxidation of low-density-lipoproteins (LDL) related with atherosclerosis, thrombotic tendencies and heart diseases Flavonoids, also present in wine by-products, were reported to reduce inflammatory reactions in the body. Additionally, polyphenols offer antiulcer, anti-carcinogenic, anti-mutagenic and antibacterial properties and also solving the environmental issues Maintain environmental balance, because large amounts of winemaking by-products are generated, which is an ecologic and economic problem in terms of storage, transformation or disposal (El Gharras, 2009).

Due to their potential health benefits, the production of grape extracts represents a good business opportunity that allows companies to target different markets. A particular emphasis should be given to the emerging profitable markets of natural ingredients for functional foods and natural cosmetics, as well as the market for dietary supplements that is easily obtained from by-products, the health and wellness consumer trend is increasing in importance, which is partly explained by populations in developed countries that are getting older and with increasing weight problems, grape pomace extracts(GPE) represent widespread uses in pharmaceutical cosmetic, and the most recent is linked to the new class of "phytosanitar bioproducts" able to control the incidence of diseases in some crops (Boussetta and Vorobiev, 2014), if used and commercialized the by-products of Ethiopian wine making has great importance in both economical and creating jobs.

Thus, data concerns the Polyphenolic content and antioxidant capacity from Ethiopian grape pomaces aiming its valorization, are still scarce. Hence, the present study aims to determine the antioxidant profile and the total phenolic content of Ethiopian grape pomace extracts. Natural extract additives have been introduced with different functions such as natural antioxidants, pigments (anthocyanins), and food preservatives which were used in different manner. Grapes and wines which can contain high levels of phenolic antioxidants have been shown to exert beneficial effects on health. Phenolic compounds can be considered as high added value by-products and the employment of low-cost industrial wastes could greatly reduce the production costs and increase the margin profit of the products.

1.4 Objective

1.4.1 General Objective

The general objective of the study was to investigate the potential for antioxidant production from wine *(Vitisvinifera L.)* by products of Red and White Wine Grape pomaces growing in Ethiopia.

1.4.2 Specific Objectives

- To analysis the proximate composition of Red and White wine grape pomace.
- To investigate the effect of process conditions namely temperature, extraction time and solvent on antioxidant total extracts yield.
- To characterize and compare Ethiopian wine grape pomace extracts (WGPE) including their total phenolic content, total flavonoid, total anthocyanin and antioxidant activity.
- To find the optimum extraction parameters for extract yield of antioxidants from red and white wine grape pomaces.
- To study the preservative effect (oxygen inhibition) of grape pomace extract on soybean oil.

1.5 Scope of the Study

This research aims at summarizing all these aspects, underlining the great opportunity of exploiting agricultural and industrial wastes for recovery of antioxidants (in particular phenols), and Extraction and characterization antioxidant (phenolic compounds) from wine-making wastes (grape pomace).

Chapter Two

Literature Review

2.1 Wine Production Globally

Wine is one of the most well-known alcoholic beverages distributed around the world, with 271 million of hectoliters (MhL) produced in 2014 according to the last bulletin emitted by Organisation Internationale de la Vigneet du Vin (OIV) (OIV, 2014) . Wine has existed since a long time as part of ancient civilizations culture. Roman and Greek cultures have begun the winemaking process some 8,000 years ago (Pinelo *et al.*, 2005). Certain chemical residues associates to wine, like tartaric acid, were discovered on 8,000-year-old pottery fragments in Greece (Cruz *et al*., 2004). Throughout time, grape exploitation has represented major advancements and important profits in ancient and current cultures. These days' three main species of grapes are distributed in the world: European grapes (*Vitisvinifera L.*), North American grapes (*Vitislabrusca* and *Vitisrotundifolia*) and French hybrids (En-Qin *et al*.,2010). However, in some Central and Eastern European countries, *Vitisrupestris*, *Vitisberlandieri* and *Vitisamurensis* species can be found, but because of their low quality- grape they are not suitable for the winemaking process (FAO, 2016).

The OIV is an intergovernmental organization of a scientific and technical nature of recognized competence for its works concerning vines, wine, wine based beverages, table grapes, raisins and other vine-based products. According to its statistics databases, the world wine production reached 271 MhL in 2014, including the following top five wine global producers: France (46.2 MhL), Italy (44.4 MhL), Spain (37 MhL), USA (22.5 MhL) and finally Argentine (15.2 MhL) (OIV, 2014).The global viticulture stage, as well as other crops, is under constant variations, mainly related to climatic factors and in other cases, due to economic policies implemented by each country. Poor weather conditions, namely mild winter, excessive humidity in spring and summer, and decreased land destined for vineyards are largely responsible for the production drops. Thus, a trend for a particular year cannot be the same for the next one. On one hand, the international scene until 2012 for countries such as China, Chile, Australia and South Africa was

greatly positive once they have experimented an increasing (from 41 % for China to over 88 % for Chile) in the total amount of wine produced between 2000 and 2012 (OIV, 2012). On the other hand, countries which were commonly recognized as references because of the high quality of their wines and the quantities produced annually, seems to have decreasing perspectives for the future. This is the case of France, Italy and Spain, which have experimented a declining tendency (28, 22, and 27 %, respectively), considering the same period of time (200-2012) in terms of wine production (OIV, 2014). The OIV also shared the ranking for the countries which actively participate in the global wine production (Table 2.1).

Table 2.1 Wine production (1000 hL excluding juice and musts)

Country	**2010**	**2011**	**2012**	**2013**	**2014**	**Ranking**
France	44,381	50,757	41,548	42,004	46,151	1
Italy	48,525	42,772	45,616	52,429	44,424	2
Spain	35,353	33,397	31,123	45,650	37,000	3
United States	20,890	19,140	21,740	23,500	22,500	4
Argentina	16,250	15,473	11,780	14,984	15,200	5
Australia	11,420	11,180	12,260	12,310	12,560	6
China	13,000	13,200	13,810	11,780	11,178	7
South Africa	9,327	9,725	10,568	10,980	11,420	8
Chile	8,844	10,646	12,554	12,846	10,029	9
Germany	6,906	9,132	9,012	8,409	9,725	10
Portugal	7,148	5,622	6,327	6,238	5,886	11
Romania	3,287	4,058	3,311	5,113	4,093	12
New Zeeland	1,900	2,350	1,940	2,480	3,200	13
Greece	2,950	2,750	3,115	3,343	2,900	14
Brazil	2,459	3,460	2,967	2,710	2,810	15

Adapted from (OIV, 2014)

2.2 Potential for Wine in Ethiopia and Awash Winery wine range

Awash Winery, which has been in existence for 70 years, is the oldest active winery in the country. Awash Winery has 117 hectares estate vineyard, which is situated majestically on a mountain plateau rising to 1,200 meters above sea level, will soon expand its vineyard planting another 180 hectares, alongside the existing vineyard. The vineyard is located in Awash Merti Jersu, only 115 km southeast of Addis Ababa. Here vines are to be found close to the equator, implying a much shorter vegetative cycle than in Europe or South Africa for example. It is possible to harvest up to twice a year from November to December, and from June to July.

The Awash Winery range consists of four wines: A white and a red made from the grapes from Awash vineyard: Kemila Medium Dry White, a slightly sweet white, mainly from Chenin blanc (80%) and Grenache Blanc. Golden color, oxidative nose with notes of beeswax. Fresh mouth with white flesh fruits. Axumit Sweet Red Wine which was produced in 2013, a blend of red Grenache (60%), Sangiovese, Petite Syrah, Gamay, Nebbiolo, Dodoma and Tinta Amarela.
The most popular Ethiopian wine with a nose of red fruit and a great acidity. And two resinous wines: Awash White wine and Gouder Red Wine.

According to recent Data Monitor figures, the global wine market is expected to increase by 3.4% between 2009 and 2014 and reach a total volume of more than 22 billion liters (equal to roughly 2.5 billion cases). This should correspond with a total market value of $291.5 billion, an increase of 10.5% over 2009 levels. Similar growth is expected in African and Middle Eastern markets. By the end of 2013, the value of wines sold in these regions is expected to be worth more than $6.7 billion. In terms of volume, this represents a total of 47.2 million liters (equal to 50 million cases). There is also evidence of increasing demand for wine within Ethiopia. Philip Parker estimates a latent demand for wine in Ethiopia of $62.5 million in 2009, 60% of which comes from Addis Ababa. Latent demand is forecast to grow to $86.71 million by 2014. Based on Data Monitor, the implicit average price per bottle of wine sold in the Middle East and Africa in 2013 will be $11.21. If we assume this implicit price, the expected latent demand translates into a potential of roughly 650,000 cases of wine purchased inside Ethiopia by 2014. However, Ethiopia is not currently prepared to take advantage of this expected growth in global and local demand. This is not due to unfavorable growing conditions. As early as the 16th century, Portuguese and Italian missionaries used Ethiopian grape varietals to make wine for church ceremonies. As recently as the 1970s, wines (particularly those made from Italian grape varietals) were produced and consumed in Ethiopia. Even today, wine grapes are grown in small quantities in several different regions. More significantly, in March of 2008, the Castel Group – France's largest wine producer – announced its $4.2 million investment to plant vineyards in the southern region of Ziway. The Prime Minister supported this development by providing the initial 300 hectares of land to Castel rent free. In response, Castel planted a 120-hectare vineyard with Merlot, Cabernet Sauvignon, Syrah, and Chardonnay, all international varietals with significant market potential.

2.3 Wine Making Process

Wine is an ancient drink that has been an important part of human societies for literally thousands of years. From its origins in ancient Greece, wine culture and the art of wine making spread throughout the ancient Mediterranean, Europe, and China. Today, wine is consumed on every continent in the world, and mainly produced in Europe, the Americas, South Africa, Australia, and New Zealand. The process of wine making has evolved throughout the centuries, and today there are thousands of wineries producing hundreds of varieties of wines.

Wine making process (Vinification) is basically a biotechnological process that transforms sugar in grape into ethanol. Yeast and appropriate fermentation conditions can provide this process happen. But in wine making process generally transformation of ethanol is not enough to obtain qualified or drinkable wine. There are lots of wine making techniques in order to combine aromatic compounds and alcohol. Most qualified wines are in balance of acidity, sugar, alcohol and phenolic compounds. There is no easy way to obtain this balance and wine making techniques are based on different biotechnological, chemical and physical methods. Enology is often defines as the science of winemaking, but in practice it combines the science, technology and engineering of the process. It is combination of interdisciplinary knowledge and principles (from chemistry, biochemistry, microbiology, chemical engineering and nutrition) which we consider to be the essence of enology (Singleton *et al*., 1999).

Wine is classified in three major categories: table wines, sparkling wines, and fortified wines. Table wines, also called still or natural wines, are consumed mostly with food, they tend to compliment the meal. Sparkling wines, for example champagne is distinguishable by its effervescence and is drunk for the most part on festive occasions such as weddings, birthdays, and during the holidays. Fortified wines, such as sherry or vermouth are most commonly Table wines are further classified by color as red, rose and white. Three of them basically have same production methods but they have some differences. Red wine production has a maceration step which takes 15-25 days with a skin contact that provides extraction of phenolic compounds into liquid phase before pressing.

2.4 Major Process Steps of Wine Making

2.4.1 Crushing and Destemming

Crushing is employed to cause berry breakage and juice release from the grapes, and ordinarily 100% of berries will be broken. It is the beginning of the juice, skin, pulp and seed contact that will influence the extent of extraction from these grape components. A secondary aspect of crushing process is the elimination of the stems from the juice and skins and the isolation and collection of them to disposal. Stems are often shredded and dispersed throughout the vineyard, dumped as solid waste or incinerated. Under some conditions partial stem removal or addition of some stems back to the must is practiced. However complete removal is generally sought (Singh, and Immanuel, 2014).

2.4.2 Fermentation

The next major step is the fermentation, in which the fermentable sugars (glucose and fructose) present in the grape juice (including any added sugar) are converted by yeasts into ethanol (ethyl alcohol) and carbon dioxide, with the generation of heat. To an extended that depends on the temperature; the fermentation also produces many of the aromatic characteristics of the finished wine. The fermentation is usually carried out in large, closed stainless-steel tanks, which are temperature controlled so as to lower the fermentation temperature as appropriate. Yeasts are unicellular microorganisms that are classified taxonomically as fungi. Yeasts have several commercial applications, and they are used also for beer brewing, baking and biomass production. Yeasts used in winemaking generally belong to the *Saccharomyces* genus, the most important species of which, *cerevisiae*, has some unique characteristics- perhaps one of the most useful ones being its tolerance to ethanol (up to 15% v/v), a very toxic compound for most other microorganisms. Red wines are fermented between 18-35°C in the presence of the skins for 3–6 days, depending on the intensity of color (anthocyanin) and dry flavor (tannins) desired. The partially fermented must is then decanted and pressed from the skins, and a secondary slower fermentation carried out to the extent required (Kammerer *et al*.,2005). Temperatures required for white wine fermentations are generally lower (rarely above 20°C) than those used for red wines, so that there is some survival of fruity esters. Hence, temperature control during white wine fermentation is much more critical. Chaptalisation is practiced by some white winemakers,

but not as frequently as is necessary for red wine production. Many white wines are not fermented out to complete dryness (i.e. they contain residual sugar), and this is best achieved by halting the fermentation, by either rapid chilling or yeast removal. After fermentation is deemed to be complete, the wine- maker has to decide whether extended lees contact and malolactic fermentation is required (Mendes *et al*., 2013).

2.4.3 Pressing

Pressing the grape mass (pomace) occurs after the free-run wine has been removed from the fermentation vat, and takes place when the winemaker decrees that the required amounts of color, flavor and tannin have been extracted. The timing can vary from 2 days to 3 weeks post-fermentation, according to wine style. Some wineries consistently leave the wine in prolonged contact with skins and, sometimes, seeds and stalks after fermentation has been completed, usually for a period of 2 or 3 weeks. This practice, which was at one time a characteristic of Bordeaux wines, is called 'extended maceration', and can often have a pronounced effect on the wine, increasing phenolic content and diminishing color. There is also some evidence that wines produced in this way have a better ageing capability (Lorrain *et al*., 2013). White wine production starts with a juice extraction by pressing immediately after crushing and draining of the grapes. Part of the juice runs out of the crushed grapes (free run juice) without added pressure and is followed by immediate pressing. Sometimes white grapes are not crushed, but immediately pressed to minimize extraction of compounds from the skins, seeds or stalks. The fermentation is carried out on the must or grape juice without the skin or pomace (Teixeira *et al*., 2014).

The most common means by which this is done is the use of belt or screw conveyors. These are often fixed in place, but in small wineries can be portable and moved into place as needed. In larger wineries, it is more usual to transfer pomace by a series of interconnecting screw conveyors that feed a group of presses and have a common dumping system (Singleton *et al*., 1999).

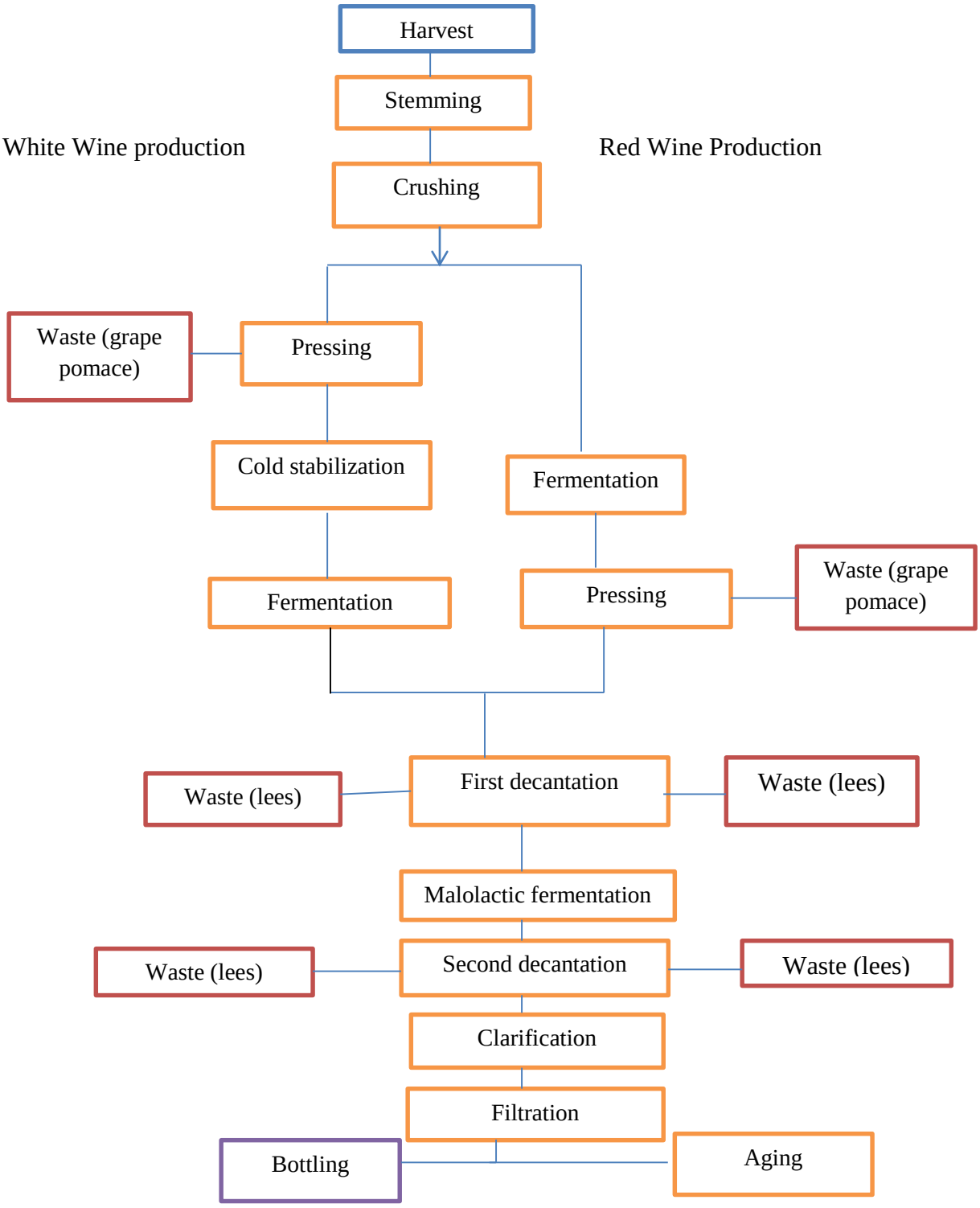


Figure 2.1 Winemaking flow chart (Atanackovic *et al.*, 2012)

2.5 Grape pomace

Grape pomace has a humidity of about 70 % and makes up for 11-15 % of grapes crushed. One ton of pomace is composed of 249 kg of "stalks", *225* kg of grape seeds and 425 kg of grape pellicles. All the major products deriving from grape pomace are depicted in Table 2.2.

Table 2.2 Chemical composition of grape pomace

Compnents		% dry weight
ashes		7.0-7.8
Extractive	dichloromethane	1.0-5.5
	water	23.7-26.4
Proteins		6.1-18.8
Tannins		13.8-15.9
Cellulose		20.8-30.3
Hemicellulose		12.5-21.0

Data from (Mendes *et al*., 2013)

After water extraction grape pomace can be used as fermentation medium to produce single cell protein (SCP) and bacterial cellulose (Rice-Evans *et al*., 1996) as well as other valuable metabolites. Solid state fermentation (SSF) is another way to produce a variety of compounds like ethanol (Rice-Evans *et al*., 1996), citric acid, gluconic acid, carotenoids, xanthan, etc. Also the possibility of enzyme production by solid state fermentation (cellulases, xylanases and pectinases) has been studied (Boussetta and Vorobiev, 2014).

Solid state fermentation essentially involves the growth of microorganisms on wet solid supports with limiting free water. This technology is interesting because is considered an appropriate approach for processes including the bioremediation of agricultural wastes and the biotransformation of crops. Moreover, SSF has been successfully applied in the preparation of new high value products, such as secondary metabolites, organic acids, pesticides, aromatic compounds, fuels and enzymes. The advantages of SSF in comparison to traditional submerged fermentation are better yields, easier recovery of products, foam absence and smaller reactor volumes. Moreover, contamination risks are significantly reduced due to the low water contents

and, consequently, the volume of effluents decreases. It has been shown that for some specific processes, particularly enzyme production, the cost of these techniques is lower and the production higher than that in submerged cultures.

Direct incorporation of grape pomace into agricultural land, a common practice, has caused serious problems since degradation products can inhibit root growth. An alternative to overcome such disadvantages and to recycle wastes is composting (El Gharras, 2009). Another suitable soil amendment is the wastewater (winery-sludge) derived from the aerobic treatment of wastewaters of the winery. Vermin compost has even more beneficial effect than normal compost (increased nitrogen, humic materials, hormones and auxins, and pH) (Chamorro *et al.*,2012). As an aerobic process, composting leads to a nitrogen mineralization and the use of earthworms in vermicomposting increases and accelerates this nitrogen mineralization rate. Winery wastes are already being used for commercial production of vermicomposting together with other material (Hagerman, 2002). Grape by-products have been used for animal nutrition (Martos *et al.*,2010). Ensiling of grape pomace in earthen pits either alone or in combination with poultry litter gave good quality silages, which can be consumed by cattle. Grape pomace composition quantity: 11-15% of grapes Composition at 70% humidity.

Grape seeds (~30% of wet pomace)

- Fibers (cellulose), pectins, mineral (K), organic acid
- Sugars (up to 150 g/Kg)
- Phenolics (tannins, anthocyanins)
- Pigments ~9 kg/t (red grape pomace)
- Tartrate ~50 to 75 kg/t

Grape skin pigment is used in wine making. The average figures for the grape pigment vary from 12 kg/t (red wine lees) to 9 kg/t (red grape pomace), the final product being in the form of a liquid concentrate with 30 g of pigments per kilogram of solution. Another proposed use is making them into a very strong permanent ink (Lapornik *et al*., 2005).

2.5.1 Grape Pomace Applications

During winemaking steps, significant amounts of waste, in their majority solids are also generated. Residues of the wine industry, including seeds, peels or skins, stalks and pulps are denominated in its whole as grape pomace.

Based on a traditional winemaking process it is estimated that per six liters of wine is generated one kg of solid waste. Thus, taking in consideration a global wine production around 271 MhL (OIV, 2014) over 4.5 million of tons of solid waste would be generated worldwide. Environmental concerns about the production and accumulation of waste worldwide are increasing. Meanwhile the European Commission, early in 2006, issued a series of regulations towards a sustainable European wine sector aimed the inclusion of minimum environmental requirements for the wine sector covering the main pressures from the sector (notably, soil erosion and contamination, the use of plant protection products, and waste management).

The current destinies for the grape pomace comprise the disposal or fertilization/compost (Laufenberg *et al*., 2003), cattle feeding, landfills, fermentation/distillation industry either for the extraction of food natural colorants and bioactive compounds (Lapornik *et al*., 2005).In certain cases, grape pomaces (mainly the seeds) are used in wood adhesives extractive processes (Ping *et al*., 2011). Some authors refer disadvantages in using grape pomace without any pre-treatment cattle feeding or in post winemaking fermentation/distillation process, due to its high polyphenols content with implications in animal nutrition and inhibition of yeasts germination (Mendes *et al*.,2013). The most recent and innovate application is associated to a new pesticide, namely "phytosanitary bioproducts" used for the control of the incidence of diseases in some crops (Boussetta, and Vorobiev, 2014). The composition of the residue of the grape has significant variations depending on grape variety and technology applied during the winemaking steps.

Generally, it consists largely in seeds and skins (or peels), and the rest is represented by stems or stalks. After fermentation step, considerable contents of polyphenols (over 10% on dry bases) are retain in grape pomace, depending on the type of grape (white or red), the part of the tissue (skins, seeds, etc.), as well as the processing conditions (e.g., contact time between skins and must) (Guendez *et al*., 2005). Regarding to its chemical composition, lignans, cellulose and tannins have been assessed previously by several authors, providing indication for content range as shown above (Mendes *et al* ., 2013).

2.5.1.1 Feed and Food Supplements

Grape pomace and other solid winery waste has been used for recovery of food ingredients, nutraceutical and functional foods (Hagerman, 2002) , that provide demonstrated physiological benefits or reduce the risk of chronic disease (grapeseed oil, β-glucans, antioxidants, etc.).
However antioxidant value depends on the recovery process. In most cases treatment procedures tend to decrease that value thus negating their potential uses as food supplements. If a cascade process is used for the recovery of more than one substance then depending on the order of recovery we can get higher quality for some products and lower for others.

By-products like grape pomace, seeds and stalks are known sources of antioxidants (phenolic acids, quercetin, flavonoids, phytoelexins and pterostylbenes, resveratrol, etc.), counteracting the effects of saturated fat and reducing the incidence of coronary heart disease mortality.
These substances also have anti-inflammatory activity, anti-carcinogenic and anti-mutagenic effects. There is considerable amount of scientific articles regarding recovery of these products from grape by-products and of new methods which deal with the improvement of techniques for the recovery and antioxidant activity of the isolated compounds, (Ping *et al*., 2011). These substances have an important added value. According to (Lu and Yeap Foo, 1999) grape seed flour has "3,000- to 5,000-fold more in antioxidant value than regular flour". Grape pomace, seeds, skin and stems extracts have effective anti-bacterial results when tested on bacteria species at a concentration of five per cent. The extracts can be used in food formulations to protect food against spoilage bacteria (Rice-Evans *et al*., 1996).

2.5.1.2 Grape seed and Grape seed oil

Cardio protective effects of grape seed proanthocyanidin are well known. Grape skins and seeds contain flavonoids (catechin, epicatechin, procyanidins and anthocyanins), phenolic acids (gallic acid and ellagic acid) and stilbenes (resveratrol and piceid). Grape seed procyanidin extract has in vivo antioxidant activity and could be as important as vitamin E in preventing oxidative damage in tissues by reducing the lipid oxidation and/or inhibit the production of free radicals.
Grape seed oil is a vegetable oil pressed from the grape seeds. It has a relatively high smoke point, approximately 216 ^{o}C, so it can be safely used for heating. In addition, it has a clean, light taste that has been described as 'nutty' and is safe for cooking food. Less grape seed oil is needed

for cooking purposes, compared with other oils. Grape seed oil is reputed to contain plentiful antioxidants, as well as substances which lower cholesterol levels. It also contains vitamin E (0.8 to 1.2 g/kg), vitamin C and Beta-Carotene. There is an unconfirmed information that grape seed oil also contains vitamin D. Grape seed oil also contains 0.8 to 1.5% unsaponifiables rich in phenols (tocopherols) and steroids (campesterol, beta-sitosterol, stigmasterol).

According to current knowledge, grape seed oil, a high linoleic (76 %) product, is the only food known to raise High density lipid HDL (good cholesterol) and Low density lipid lower LDL (bad cholesterol). Low level HDL is also a risk factor for impotence. Linoleic acid is one of two essential fatty acids people cannot manufacture themselves. Linoleic acid is an omega-6 fatty acid. Grape seed oil is a preferred cosmetic ingredient for damaged and stressed tissues, for possessing regenerative and restructuring qualities which allow a better control of skin miniaturization and protection.

Table 2.3 Grape seeds composition

Quantity (30% of wet pomace)	Composition (%)
Water	25-45
Sugars,polisaccharides	34-36
Organic acid	2-7
Oils,fatty acids	13-20
phenolics	4-6
Nitrogen substance	4-6
Minerals,inorganic	2-4
Vitamins(E,A,C,B1,B2,B5,B6,B9),β-carotene	-

2.6 Antioxidants

A substance, when present at a low concentration compared with that of an oxidizable substrate, inhibits oxidation of the substrate. Oxidation is a chemical reaction that transfers electrons from the substance to an oxidizing agent. Oxidation reactions can produce free radicals.

In turn, these radicals can start chain reactions that deteriorate foods. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation

reactions, so that extends the induction time of the foods. They do this by being oxidized themselves, so antioxidants are often called reducing agents such as thiols, ascorbic acid or Polyphenols. Addition of antioxidants after the end of this period tends to be ineffective in retarding rancidity development. The induction time (IT) is very sensitive to small concentrations of components that shorten it; the pro-oxidants, or lengthen; antioxidants. Metal ions are the most important prooxidants in foods, whereas antioxidants include compounds that act by radical scavenging, metal chelating or other mechanisms (Kammerer *et al*., 2005).

Antioxidants can inhibit or retard oxidation in two ways: either by scavenging free radicals, in which case the compound is described as a primary antioxidant, or by a mechanism that does not involve direct scavenging of free radicals, in which case the compound is a secondary antioxidant. Primary antioxidants include phenolic compounds such as vitamin E (α-tocopherol).These components are consumed during the induction period. Secondary antioxidants operate by variety of mechanisms including binding of metal ions, scavenging oxygen, converting hydro peroxides to non-radical species, absorbing UV radiation or deactivating singlet oxygen. Normally, secondary antioxidants only show antioxidant activity when a second minor component is present. This can be seen in the case of sequestering agents such as citric acid which are effective only in the presence of metal ions, and reducing agents such as ascorbic acid which are effective in the presence of tocopherols or other primary antioxidants (Makris *et al*., 2007).The most important mechanism of antioxidants is their reaction with lipid free radicals, forming inactive products. Additives with this mechanism are antioxidants in the proper sense. Usually, they react with peroxy or alkoxy free radicals, formed by decomposition of lipid hydro peroxides. Other inhibitors stabilize lipid hydro peroxides, preventing their decomposition into free radicals. Some substances called synergists demonstrate no antioxidant activity in themselves, but they may increase the activity of true antioxidants (Angshuman and Chiranjit, 2012).

2.7 Antioxidants and Health

Oxidative DNA damage may be a major risk factor for the development of cancer, so that dietary antioxidants able to decrease such damage in *vivo* would be expected to have an anticancer effect (Huang *et al*., 2005). In the last few decades, several epidemiological studies have shown that a dietary intake of foods rich in natural antioxidants correlates with reduced risk of coronary heart disease; particularly, a negative association between consumption of polyphenol-rich foods and cardiovascular diseases has been demonstrated. This association has been partially explained on the basis of the fact that polyphenols interrupt lipid peroxidation induced by reactive oxygen species. A large body of studies has shown that oxidative modification of the low-density fraction of lipoprotein is implicated in the initiation of arteriosclerosis. More recently, alternative mechanisms have been proposed for the activity of antioxidants in cardiovascular disease, which are different from the simple shielding of low density lipoprotein from reactive oxygen species, induced damage.

Several polyphenols recognized for their antioxidant properties might significantly affect cellular response to different stimuli, including cytokines and growth factors (Lu and Yeap Foo, 1999). Dietary consumption of polyphenols is associated with a lower risk of degenerative diseases.
In particular, protection of serum lipids from oxidation, which is a major step in the development of arteriosclerosis, has been demonstrated. More recently, new avenues have been explored in the capacity of polyphenols to interact with the expression of the human genetic potential.
The understanding of the interaction between this heterogeneous class of compounds and cellular responses, due either to their ability to interplay in the cellular antioxidant network or directly to affect gene expression has increased (Moure *et al*., 2001). Foods rich in antioxidants may afford a degree of protection against free radical damage not only in foods, but also in the human body, protecting against cardiovascular diseases, damage of nucleic acids, and other deteriorative processes. The absorption of tocopherols and carotenoids into the blood stream is well known, but much less has been published on the fate of other antioxidants and their reaction products. Some antioxidants may not be absorbed in the intestinal tract at all, even when they are active in foods (Boussetta and Vorobiev, 2014).

2.8 Polyphenolic Compounds and Antioxidant Properties

Phenolic compounds (or just polyphenols) represent a wide family of compounds, including various groups of molecules classified as plant secondary metabolites. Phenolics have been considered the most important, numerous and ubiquitous groups of compounds in the plant kingdom (Naczk and Shahidi, 2004). More than 8,000 different compounds have been identified and the number is still growing (Ignat *et al*., 2011), including complex chemical structures which exert diverse biological functions. A first classification can be made based on their solubility. The water-soluble polyphenols comprise compounds such as phenolic acids, phenylpropanoids, flavonoids and Quinone's; whilst those which are water-insoluble include: condensed tannins, lignins and cell-wall bound hydroxycinnamic acids (Huang *et al*., 2005).

Konczak (2004) classified these bioactive compounds according to the number of phenol rings they contain in: phenolic acids, stilbenes, flavonoids, lignins and tannins (Konczak, 2004). All these groups present one or more hydroxyl groups directly attached to an aromatic ring, conferring the phenolic characteristics. Flavonoids, the most important single group of polyphenols, include 13 subclasses with more than 5,000 different compounds present mainly in fruits and plants (Bravo, 1998).Among these subclasses, compounds namely, chalcones, dihydrochalcones, aurones, flavones, flavonols, dihydroflavonol, flavanones, flavanols, flavandiol, anthocyanidins, isoflavoinids, bioflavonoids, and proanthocyanidins or condensed tannins, can be found in food sources. The flavonoids basic structure consists in a common diphenylpropanes (C_6-C_3-C_6) skeleton with an essential structure consisting in two aromatic rings, A and B joined by a 3-carbons bridge, usually in the form of an oxygenated heterocyclic ring C, as shown in Figure 2.2. Variations in the substituent groups in the ring C give the major flavonoid aforementioned subclasses. Moreover, flavonoids can be found in a non-glycosylated form (aglycone) as occasionally occur in plants, or most commonly attached to a sugar molecule (glycoside) (Bravo, 1998; Ignat *et al*., 2011).

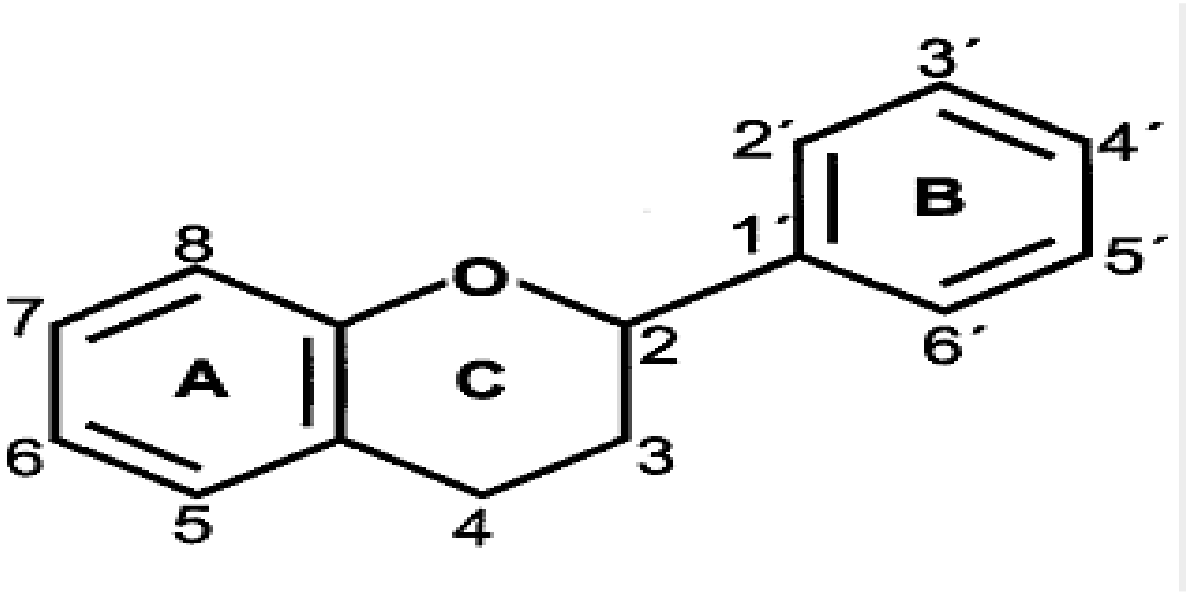


Figure 2.2 Basic structures and numbering of the flavonoid nucleus, Source: (Bravo, 1998)

Considering the importance of flavonoids compounds, a brief description, highlighting the most remarkable characteristics of main subclasses, is given bellow. Flavonols, the most ubiquitous flavonoids in foods, include as main representative compounds, kaempferol and quercetin.

They present strong antioxidant properties, mainly quercetin, through the free radical scavenging activity. Quercetin (Figure 2. 2) presents the three fundamental criteria to be considered a strong free radical scavenger as follow:

- The *O*-dihydroxy structure in the B ring, which confers higher stability to the radical form and participates in electron delocalization;
- The 2,3 double bond in conjugation with 4-oxo function in the C ring is responsible for the electron delocalization from the B ring, in other words, the antioxidant tendency is associated to this structure regarding the resonance effect of the aromatic nucleus;
- The 3- and 5-OH groups with 4-oxo function in A and C rings are required for maximum radical scavenging potential (Rice-Evans *et al*., 1996).

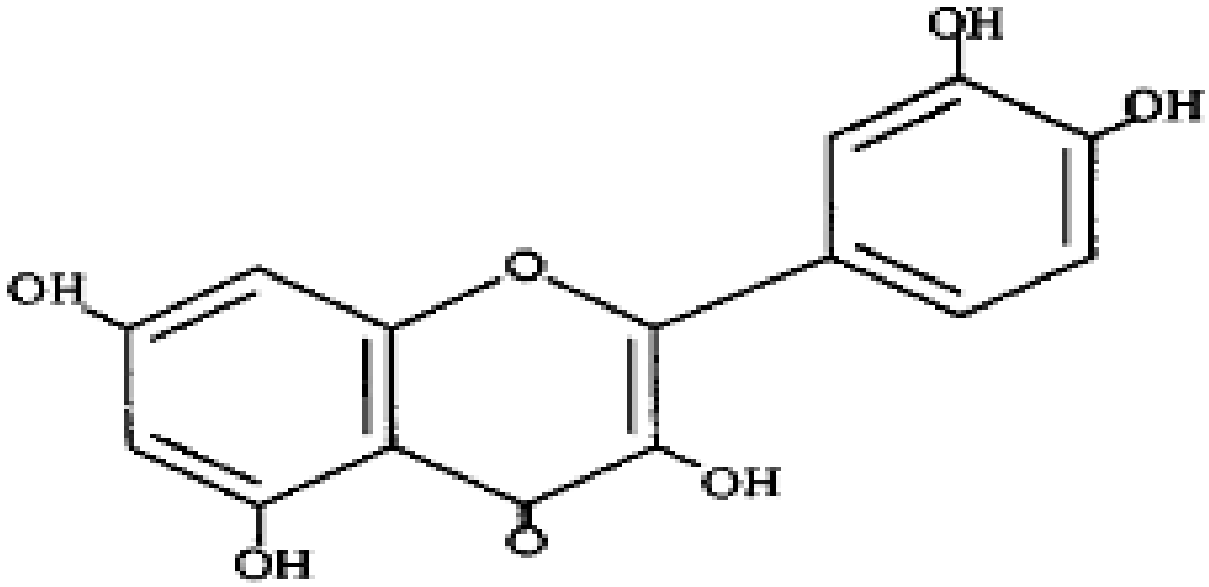


Figure 2.3 Chemical structure of quercetin, Source: (Rice-Evans *et al*., 1996)

Good flavonols sources are onions, curly lake, blueberries, passion fruit, pomegranate and broccoli. Red wine and tea also contain up to 45 mg flavonols per portion (El Gharras, 2009) . Flavones are much less common than flavonols in fruit and vegetables. Significant quantities are found in the polymethoxylated form as tangeretin, nobiletin and sinensetin in the skin of fruit citrus (essential oil of mandarin, for example). The only important edible sources of flavones identified till these days are parsley and celery. These polymethoxylated flavones are the most hydrophobic flavonoids (Makris *et al*., 2007). Flavones are found in tomatoes and certain aromatic plants such as mint, but they are present in high concentrations only in citrus fruit. They can appear in the aglycone form like naringenin in grapefruits, hesperidin in oranges, and eriodictyol in lemons. Nevertheless, the most common forms appear generally as glycosylated (*O*- or *C*-glycosides) by a disaccharide in certain cases or by a rutinose in others. High flavanones concentrations are found in the solid parts of citrus fruit, particularly the albedo (the white spongy portion) and the membranes separating the segments (Bravo, 1998;El Gharras, 2009). Isoflavones, such as daidzein and genistein, with ring B of the flavone molecule attached to the carbon 3 of the heterocycle, especially occur in legumes (Bravo, 1998). According to El Gharras isoflavones are provided only by soybean derived products.

They can be present as aglycones or glycosides, depending on the soy preparation. Soya and its processed products are the main source of isoflavones in the human diet (El Gharras, 2009). Compounds lies in the fact that certain physiological effects are attributed to their similar structure to estrogens like β-estradiols. Additionally, for this reason they are sometimes described in the literature as "phytoestrogens" (Ignat *et al*., 2011). Flavonols comprise two types of associations that may exist between compounds. On one hand, it may exist in the form of monomers like catechins and On the other hand, it is also possible to find them in the polymer form reveling a more complex structure (proanthocyanidins). Some sources for catechins are many types of fruits like apricots and sweet cherry, some beverages such as red wine, although green tea and chocolate are the richest sources (Makris *et al*., 2007). Catechin, epicatechin and gallocatechin are the monomeric constituents of the condensed tannins, although they are also commonly found as free monomers (Bravo, 1998).

Catechin and epicatechin are the main flavonols in fruit, whereas gallocatechin, epigallocatechin, and epigallocatechin gallate are found in certain seeds of leguminous plants, in grapes, and more importantly in tea (Angshuman and Chiranjit, 2012). Likewise, most of the polyphenolic compounds, catechins and their esters, particularly epigallatocatechin gallate present in the green tea, have shown anticarcinogenic actions in human and animal tissues (Rice-Evans *et al*., 1996). According to a research work by Boussetta and Vorobiev,(2014) the intake of catechin originating from fruits, but not from tea, was associated to a lower risk of cancer of the upper-digestive tract (Goupil *et al*., 2012).Finally the last groups of compounds belonging to flavonoids are the water soluble vacuolar pigments that may appear as red, purple, or blue depending on the pH, are the anthocyanins (Ignat *et al*., 2011).

The term anthocyanin refers to the glycoside of anthocyanidins (Bravo, 1998).The anthocyanidins consist of an aromatic ring A bonded to an heterocyclic ring C that contains oxygen, which is also linked by carbon-carbon bond to a third aromatic ring B (Konczak, 2004). Up to now there are reports of more than 500 different anthocyanins and anthocyanidins (Castañeda *et al*., 2009), although the most frequently reported in the plant kingdom are the following six anthocyanins: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Among the sugars linked to the anthocyanidins forms are the monosaccharides: glucose, galactose, rhamnose and arabinose (Ignat *et al*., 2011).

The color of the anthocyanins is largely conditioned by the substitution pattern of the ring B of the anthocyanidins, the pattern of glucosylation and the degree and nature of esterification of the sugar with aliphatic or aromatic acids and by the pH, temperature, and type of solvent and presence of co-pigments (Sáyago-Ayerdi *et al*.,2009). The major antioxidant activity of the anthocyanins can be ascribed to the reducing power of the *O*-dihydroxy structure in the B ring (Rice-Evans *et al*., 1996).

This significant property plays a vital role in the prevention of neuronal and cardiovascular illnesses, cancer and diabetes, among others. For example, previous study has showed that anthocyanins from wine and grape skin inhibited phosphodiesterase-5 activity, which reduced the risk of cardiovascular diseases by vasorelaxation (En-Qin *et al*., 2010). In fact, the group of flavonoids was found to be very effective scavengers of free radical concerning *in vitro* assays, showing important antioxidant activity due to their high redox potential which allows them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. Metal chelating properties were also described for flavonoid compounds (Huang *et al*., 2005).Regarding their importance, the second most remarkable Polyphenolic group is the phenolic acids, which represent one- third of the polyphenols present in the human diet. They can be found not only in bound forms but also in the free form in plant (Ignat *et al*., 2011).

Nevertheless, their methyl and ethyl esters, also with their glycosides occur very commonly as bound forms (Bravo, 1998). There are two subclasses for the phenolic acids: hydroxycinnamic and hydroxybenzoic acids. The presence of one carboxylic group in the structure confers their acidic character. Phenolics with C_6-C_1 skeleton such as gallic, vanillic, syringic and hydroxyl benzoic acids, and their aldehydes are quite common in higher plants and ferns. The most important phenylpropanoids (C_6-C_3 skeleton) are the hydroxycinnamic acids like *p*-coumaric, caffeic, ferulic and sinapic and their derivatives. Both these groups (phenylpropanoids and more simple phenols) are usually linked by covalent bonds to cell wall polysaccharides or to the so called lignin core (Bravo, 1998; El Gharras, 2009). Tannins represent polyphenols of intermediate to high molecular weight compounds. They are mostly present in fruits in the polymeric form and they are responsible for the astringency of tannin-rich foods, due to their ability to precipitate the proteins present in the saliva (Bravo, 1998). Tannin group could be divided into two sub-classes: hydrolysable and condensed tannins (also called proanthocyanidin). Hydrolysable tannins are gallic acid and its dimeric condensation product, hexa hydroxydiphenic

acid, esterified to poly ol, which is mainly glucose. Further esterification or cross linked oxidation reactions take place to yield more complex hydrolysable tannins (Hagerman, 2002) . They are found in fruits and as their name indicates, these compounds are easily hydrolyzed in acid or alkali medium, for hot water or enzymatic action, giving as result polyhydric alcohol and phenyl carboxylic acid. One of the most representative compounds belonging to hydrolysable tannins is the tannic acid (Bravo, 1998).On the other hand, condensed tannin or proanthocyanidin, pertaining to the family of flavonoids, consist in monomeric units of flavan-3-ol (catechin, epicatechin, etc.) with a flavan-3,4-diol as its precursor. Pathways involved in their biosynthesis although are well understood, the steps leading to condensation and polymerization have not been elucidated yet (Ignat *et al*., 2011). For this reason, most of the published literature refers to oligomeric proanthocyanidin like dimmers, trimmers. However, proanthocyanidin can reach high polymerization degrees (Bravo, 1998). The properties behind the chemical structure for the tannins are mainly linked to potential metal chelators, protein precipitating agents and biological antioxidants (Ignat *et al*., 2011).

One important source for the condensed tannins is grapes, where they are mainly localized in hard part of the fruit, like seeds (El Gharras, 2009). Since the chemical point of view, stilbenes are phenylpropanoids-derived compounds characterized by a 1, 2-diphenylethylene skeleton (C_6-C_2-C_6). They are not abundant in the human diet. Resveratrol is probably the most representative compound belonging to this group and exists in red skin grape, peanuts and berries (Ignat *et al*., 2011). It can be found in both *cis*- and *trans*-resveratrol (3,5,4'-trihydroxystilbene) isomers, and also as resveratrol-3-*O*-β-Dglucopyranoside (piceid), piceatannol (3,4,3',5'-tetrahydroxy-*trans*-stilbene) and resveratrol dimmers in grapes (Fontana *et al*., 2013). It has been intensively studied all over the world due to its beneficial health properties, linked to circulatory system, prevention the development of degenerative diseases like arteriosclerosis and also anticarcinogenesis (El Gharras, 2009). Because resveratrol is synthesized as response to insect attack, injury and fungal infection (particularly *Botrytis cinerea*), it represents a phytoalexin substance (Atanackovic *et al*., 2012). Stilbenes can also occur in oligomeric and polymeric forms, so-called *viniferins*.

They are induced by oxidative polymerization of the monomer resveratrol through the activity of a peroxidase (Gülçin , 2010). Lignans or phytoestrogens represent one of the major groups of polyphenolic compounds with a chemical behavior which allow oestrogen-like activities.
In other words, they are converted into certain compounds (enterodiol and enterolactone) in the intestinal lumen which exhibit both oestrogenic and antioestrogenic properties. Lignans are generated by oxidative dimerisation of two phenylpropane units. In most of the cases they are present in nature in free form and very few can be seen as glycoside derivatives (Ignat *et al*., 2011). Flaxseed, sunflower and certain cereals such as rye, oats and barley are the major source of lignans in the human diet (Makris *et al*., 2007). Particularly, the scientific areas have been expressed interest in lignans research due to potential applications in cancer therapies and because it seems to exist a relationship between the consumption of whole-grain cereals, the major source of lignans, and the risk reduction of various cancers (Sáyago-Ayerdi *et al*., 2009).

2.9 Classes of Natural Antioxidants

Antioxidants can be classified as liposoluble or hydrosoluble antioxidants. Liposoluble antioxidants are located mainly on membranes or are associated with lipoproteins, while hydrosoluble antioxidants circulate more freely in the blood. Vitamin E, which is highly liposoluble, has a particular affinity for lipoproteins, whereas vitamin C, which is highly hydrosoluble, circulates freely with minimal protein binding (Chamorro *et al*.,2012). Functionally, antioxidants can be grouped according to their preferential localization and it is the formulation of antioxidant combinations. This classification identifies antioxidants as follows:

- Membrane antioxidants: these are represented by vitamin E, β-carotene, vitamin A, and are known also as lipophilic antioxidants. They have an affinity for membranes of cells and lipoproteins (low-density lipoprotein, very low density lipoprotein, high-density lipoprotein).
- Circulating antioxidants: these consist of vitamin C, amino acids, and polyphenols, which are also known as hydrophilic antioxidants. They are not heavily bound to proteins and may circulate freely in body fluids.
- Cytosol antioxidants: these are produced by cells. Members of this class are lipoic acid, squalene, coenzyme. They are intermediates for the synthesis of endogenous molecules or macromolecules (cytochromes).

- System antioxidants: these are trace metals (such as Se and Zn) or amino acids (such as L-cysteine) (Cruz *et al*., 2004). Another group of antioxidants is classified based on the direct or indirect activity of these compounds.

2.10 Sources of Antioxidants

Plant foods provide a wide variety of dietary antioxidants, such as vitamins C and E, carotenoids, flavonoids and other phenolic compounds. Natural antioxidants such as vitamin C and E are used as a means of enhancing biological functions and improving the stability of lipid and lipid-containing products. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*butylhydroquinone (TBHQ) and propylgallate (PG) are also used but are under strict regulation because of potential health hazards (Huang *et al*., 2005).

Due to potential health hazards of synthetic antioxidants, researchers have embarked on a continuous search for natural antioxidants as alternatives to synthetic ones for application in food products and cosmetics.

2.11 Extraction of Polyphenols from Grape Pomace

In a context where the world production of wine each year generates tons of waste liable to be used in obtaining valuable bioactive compounds, a large number of works have been published in relation to the utilization of by-products (Fontana *et al*., 2013). Usually, all the extractives procedures start with a sample pre-treatment including oven or freeze drying, ground to finer powder or crushing fresh tissues (Fontana *et al*., 2013). Extraction procedures existing in the literature range from conventional methods of solvent mediated solid-liquid extraction, through methodologies based on supercritical properties of fluids (supercritical liquid and supercritical fluid extraction) to the most emerging technologies such as enzymatic hydrolysis treatments, ultrahigh pressure systems, high voltages electric discharges and pulsed ohmic heating. Regarding conventional methodologies, there are extractive solvent systems based on solid-liquid system transfer phenomena which are used during separation operations. After applications of these methodologies, phenolic-rich crude extracts are obtained (not individual or compounds families). Thus, the extraction efficiency can be improved by changes in concentration gradients, diffusion coefficients, solvent type, particle size, temperature, and extraction time as well as the presence of interfering substances in the matrix.

The solvent type (polar or hydrophobic nature) has been pointed out as one of the most important variable in the extraction efficiency of the process (Fontana *et al*., 2013).
The extraction yields in terms of the total polyphenol content can be enhanced with the assistance from as a simple and economic ultrasound technology technique (Luque -de Castro and Priego-Capote, 2007).

The use of supercritical fluids resulted in advances in the extractive process as it takes advantage of the ease with which manages to penetrate the matrices of solid waste under conditions that avoid the presence of light and oxygen, also improving process efficiency (Wells, 2003). Among the most common solvents used in supercritical fluid extraction, supercritical carbon dioxide presents advantages due to its relatively low temperature during the extraction steps, avoiding the degradation of the valuable bioactive compounds. The application of supercritical CO_2 is enhanced with the addition of small amounts of solvents (co-solvents or modifiers) such as methanol and ethanol, thus improving contact with more hydrophilic compounds (Wang and Weller, 2006). The choice of a particular modifier is restricted to the subsequent use of the extracted compounds. Although this technique still represents a promising extractive methodology, further studies regarding to costs involved should be done. Accelerated solvent extraction, also known as pressurized fluid extraction or pressurized liquid extraction, uses solvent at high temperature (100-180 °C) and pressure (1500-2000 psi) in order to improve the extraction of bio actives compounds from solids matrixes (Fontana *et al*., 2013).
Recently Rockenbach *et al.* (2012) proposed a promising new approach (at 25 °C) once the properties of the polyphenols are influenced by high temperatures (Rockenbach *et al*.,2012). In other research areas, enzymatic hydrolysis procedures have been performed in order to improve the extraction of bioactive compounds. Mixtures of pectinolytic and cell-wall polysaccharide degrading enzymes in aqueous medium (Kammerer *et al*., 2005), carbohydrates (cellulolytic and pectinolytic activities) and tannase (Chamorro *et al*., 2012) and more recently, pectinase, cellulase and tannase (single and blended treatments) (Fernández, 2015) were successfully exploited. Nevertheless, further studies are needed to identify more specific enzymes with potential use in the releasing of polyphenols from grape pomace. This is of particular relevance since several *in vitro* antioxidant assays showed that the bound phenolic fraction demonstrated a significantly higher antioxidant capacity than free and esterified phenolics.

In the same context of new trends for the extraction of bioactive compounds, other technologies like high voltage electric discharge and pulsed ohmic heating are under study. High voltage electric discharge, combining temperature, different solvents, energy and exposition time, was demonstrated to be useful for the particle fragmentation and cell structure damage accelerating the extraction of intracellular compounds. Still, studies regarding to the associated costs and design at a commercial level are required (Boussetta and Vorobiev, 2014).

Pulsed ohmic heating is an emerging technology which allows high cell membrane permeabilization of the materials under study, with low energy consumption combining electrical and thermal treatments. El Darra *et al*. (2013) believe that this methodology is promising for future application in the valorization of pomace from fruits and vegetables without hydro alcoholic solvent use (El Darra *et al*., 2013).

2.12 Polyphenolic Compounds from Grape Pomace and Separation

2.12.1 Characterization and Evaluation of Antioxidant Properties

Emerging attention to trends related to the grape pomace bioactive recovery processes lead to the exploration of accurate techniques to assess their antioxidant properties. Thus, there is an increasing interest in high-throughput techniques, automatic and rapid assessment methodologies to evaluate the antioxidant properties in complex matrixes like grape pomace. Different efforts were made in order to study, classify and propose a general guideline about the current involved antioxidant methodologies and assays (Makris *et al*., 2007).

Generally speaking, the quantification of the polyphenols in grape pomace starts by evaluating the Total Phenolic Content (TPC), with forward steps consisting in an evaluation of the antioxidant capacity (through more than one single methodology) and a complementary identification and quantification of the individual phenols. The simplest method for a fast estimation of TPC is the measurement of absorption at 280 nm (in a suitably diluted sample). The second method most commonly used for TPC assessment is the Folin−Ciocalteu's assay (Fontana *et al*., 2013), also named Folin-Ciocalteu's reducing assay (FCR). The FCR actually measures the sample's reducing capacity, but this is not reflected in the name "total phenolic assay" (Huang *et al*., 2005). It has been strongly recommended to use at least two methods for

the assessment of antioxidant properties when working with complex matrixes (Schlesier *et al*., 2002).

It is also advantageous to select methods that are commonly accepted, validated and standardized, with a large body of comparable data available in the literature (Magalhães *et al*., 2008).There are certain requirements or criteria which must be followed in order to select and accurately standardize an "ideal" methodology for the antioxidant capacity assessment:

i. measures chemistry actually occurring in potential applications;
ii. utilizes a biologically relevant radical source;
iii. simple;
iv. uses a method with a defined endpoint and chemical mechanism; (v) instrumentation is readily available;
v. good within-run and between-day reproducibility;
vi. adaptable for assay of both hydrophilic and lipophilic antioxidants and use of different radical sources;
vii. Adaptable to "high-throughput" analysis for routine quality control analyses (Prior *et al*.,2005).

Particularly when working with grape pomace many spectrophotometric assays have been proposed: DPPH• (2, 2-diphenyl-1-picrylhydrazyl) assay, ORAC (Oxygen Reactive Absorbance Capacity) assay, TEAC (Trolox Equivalent Antioxidant Capacity) and TBARS (Thiobarbituric Acid Reactive Substances) assay. Besides, as some polyphenols are also effective as chelators of transition metal ions (which may induce Fenton-type oxidation reactions in their free states (Rice-Evans *et al*., 1996), assays based on this antioxidant property like iron(II) chelating ability (ICA) assay, have been applied. In DPPH• assay, the purple chromogen radical 2,2′-diphenyl-1-picrylhydrazyl (DPPH•) is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine, following the decrease of absorption at 517 nm. Results are typically expressed in Trolox equivalents (TE). In ORAC assay a peroxyl radical is thermally generated *in situ* from AAPH (2, 2- azobis -2-amidino-propane) dihydrochloride) which reacts later with a fluorescent probe (typically fluorescein or phycoerythrin). The antioxidant presence avoids the fluorescent probe degradation, prolonging its emission upon time.

Chapter Three

Materials and Methods

3.1 Materials

3.1.1 Raw Material and Equipment

Red and white wine grape pomace (RWGP and WWGP) were kindly supplied by Awash Winery Factory located in Addis Ababa, Ethiopia. Grape pomace was frozen and transported to laboratory of Food Technology and Process Engineering, Wollega University.

The equipment used for the experiment were: Computerized UV/Vis Spectrophotometer (model UV-752), condenser, sieves, volumetric flasks, water bath shaker(Nutronics, model PMTC-3046), vacuum rotary evaporator (Fisatom, model 801) ,drying oven (Tecnal, model DHG-9203A), furnace(Gallenkamp, modelSX-2.5-10), coffee grinder (Mouliner,AR1044), tray, centrifuge(Labtech, model AVI-558) refrigerator(WestPoint, model WRES-358.X), soxhlet, Erlenmeyer flasks, small bottles (glass jars), sensitive balance (Electronic balance,FA2004B), pH meter, measuring beaker, quantitative filter paper which is equivalent to what man No. 42., Laboratory thermometer, spoon, pipette, micropipette, vortex mixer(DLAB ,model MX-SVB6F035754), test tubes, cuvettes (1cm, 2ml plastic or glass), racer, Kjeldahl flask (model KDN-102F).

3.1.2 Chemicals

Ethanol, Methanol, Folin Ciocalteu Reagent, Gallic acid, catechin, sodium carbonate, ascorbic acid, aluminum chloride ($AlCl_3$), DPPH, Sodium nitrite ($NaNO_2$), Sodium hydroxide (NaOH), were used during the investigation.

Ethanol and methanol were bought from Charkos Market Center, but all other listed chemicals were available and used in the Awash Winery and Center of Food Science and Nutrition, College of Natural Science, AddisAbabaUniversity as well as in Department of Food Technology and Process Engineering, Wollega University.

3.2 Frame Work of the Experiment

The research frame work was conducted as shown below in the following general diagram which shows all activities performed during the work.

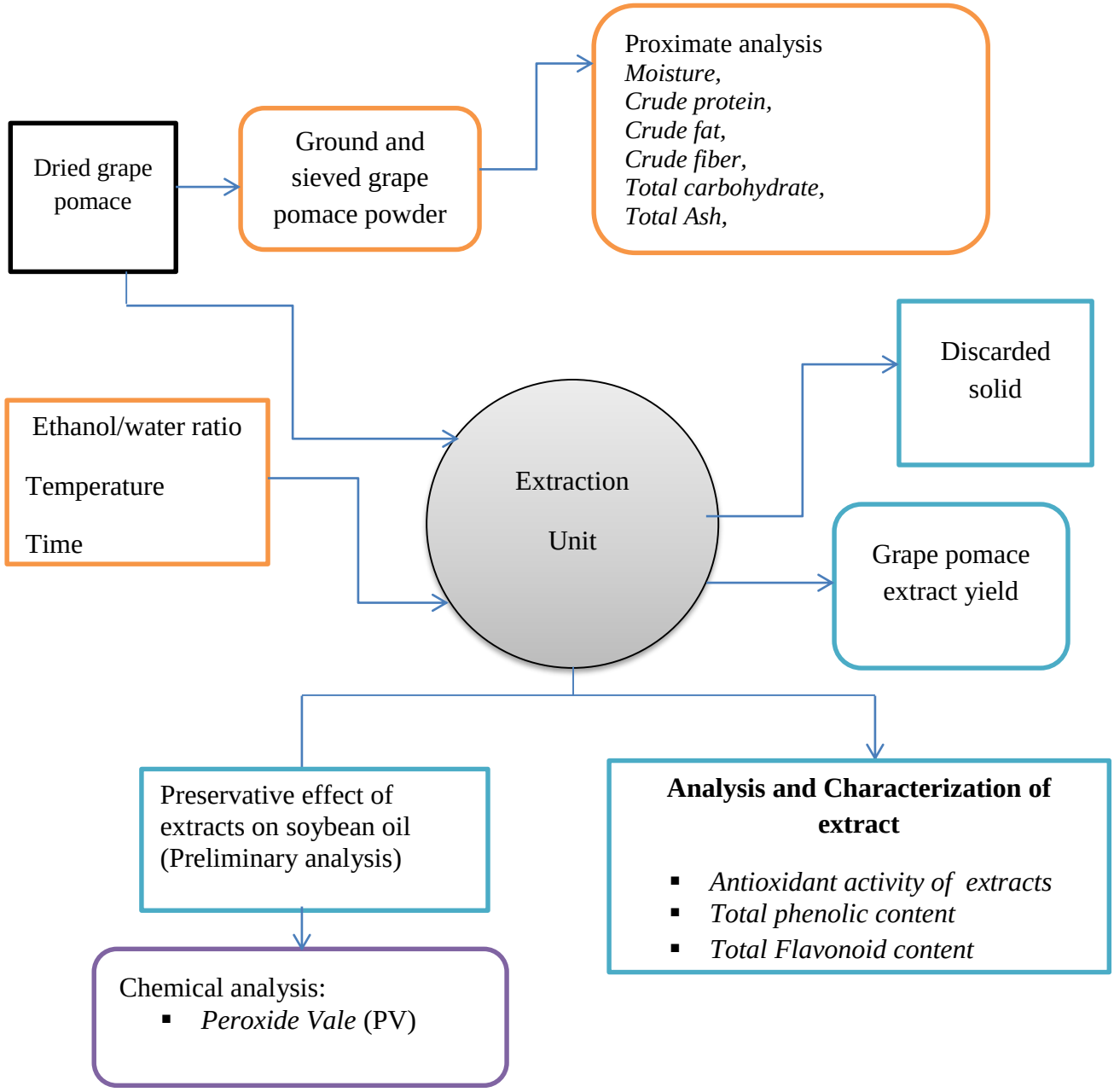


Figure 3.1 Experimental framework of the thesis

3.3 Methods

3.3.1 Preparation of the Grape Pomace Extracts

Red and White Wine grape pomaces and a given amount of stems (5-6% of the whole bunch that comprised the pomace) were obtained after the last pressing step, in January 17/2017 packaged into a dark polyethylene-bag, labeled, frozen immediately and transported to the laboratory. Samples were defrosted at room temperature prior efficiently mixture to guarantee a representative proportion of seeds and skins. Then, a portion of 500 g sample was placed on a tray and dried in an oven. Oven drying condition was 55 ºC with forced air. The final point of the drying was assessed by sampling and evaluating the moisture content by weighing differences till reaching less than 5% (w/w) (in triplicate). Finally, the dried material was stored in dark-packaged polyethylene and stored at -18 º C and the grinding, was performed in the following day.

A coffee grinder was applied to reduce the size of particles within intervals of a gap of fifteen seconds to prevent thermal stress of the material. The entire procedure was performed protecting the material from the light. Extraction was carried out as described by Shirahigue *et al*. (2010) with a few modifications.

3.3.2 Setting Extraction Parameters

For this research the independent variables or factors which have a direct effect on the dependent variables or response (total yield) were extraction solvent (Ethanol/water ratio) at concentration of 60:40,75:25 and 90:10 v/v, extraction temperature (30 ,45 and 60°C), and extraction time (60 , 90 and 120 minutes) for actual variable levels. For each factors, an experimental range was adjusted based on the result from literature. These three factors: extraction solvent concentration of ethanol/water ratio, extraction temperature and extraction time is selected as independent variables, because of their influence on antioxidant properties of phenolic extracts in plant materials (Wijngaard *et al*., 2012). In this study, the particle size was controlled as constant at interval of 2-3mm by passing through sieve.

The 2-3mm size is optimal for extraction, while smaller particles may become slimy during extraction and create difficulty during filtration (Sukhdev *et al*., 2008).

3.3.3 Extraction of Antioxidant Constituents

Experimental Procedure: For each solvent ratios, time and temperature, from the source, 20g dried and ground grape pomace was extracted in a water bath shaker (Nutronics Scientific Co., model PMTC-3046) with a 60:40,75:25 and 90:10 (v/v) ratio of ethanol/water at temperature of 30,45 and 60 °C for 60,90and 120 minutes in a conical flask. The liquid extract was separated from solids through Whatman No. 42 filter paper and further clarification by using centrifugation at 5000rpm for 15 minutes at room temperature. The filtrates were dried by vacuum rotary vapor evaporator at 65°c. The dried extracts were weighted to analyze the total yield, (Chamorro *et al*., 2012).

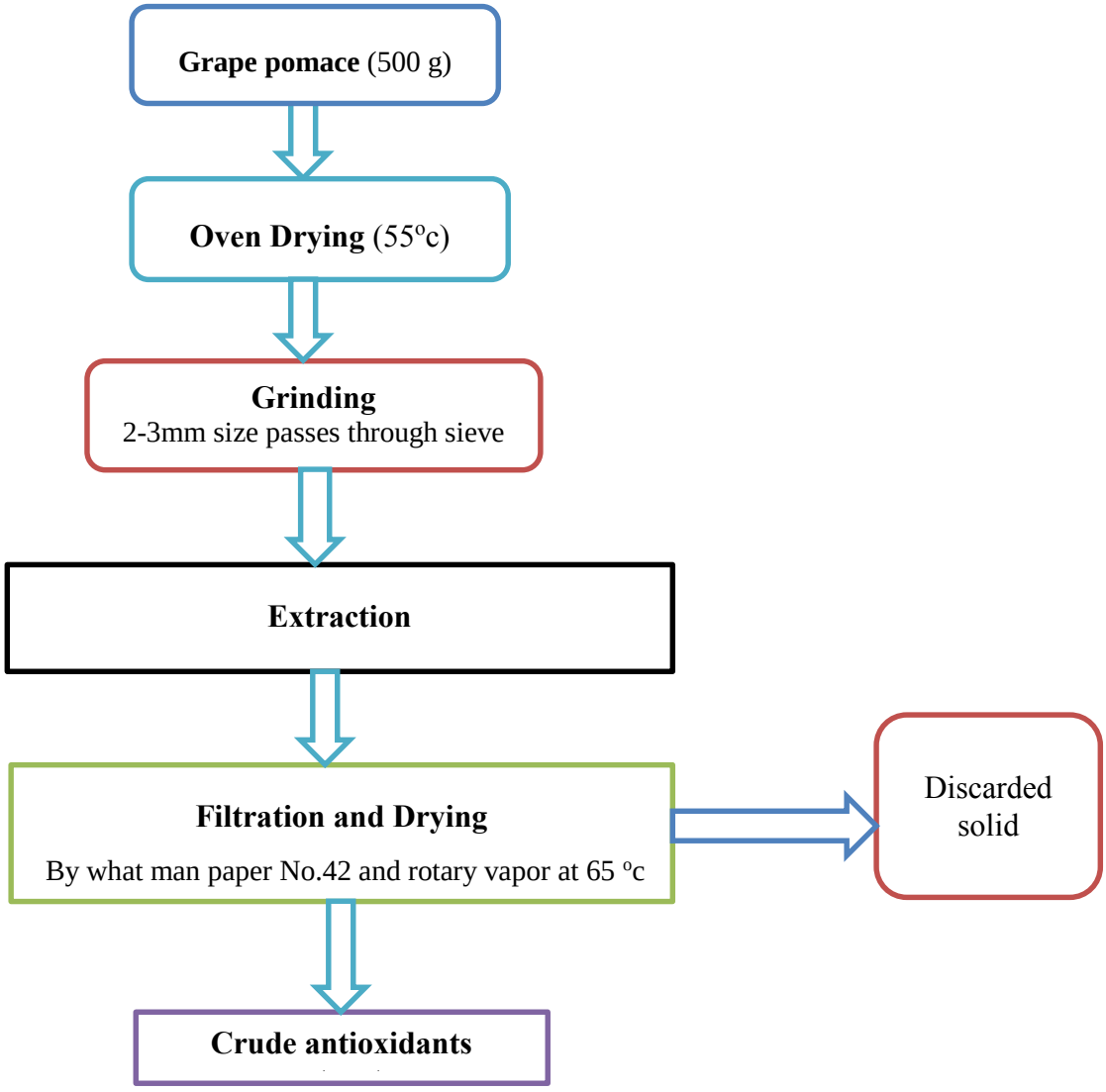


Figure 3.2 Equipment setup for extraction of antioxidants from Wine grape pomaces (Shirahigue *et al*., 2010)

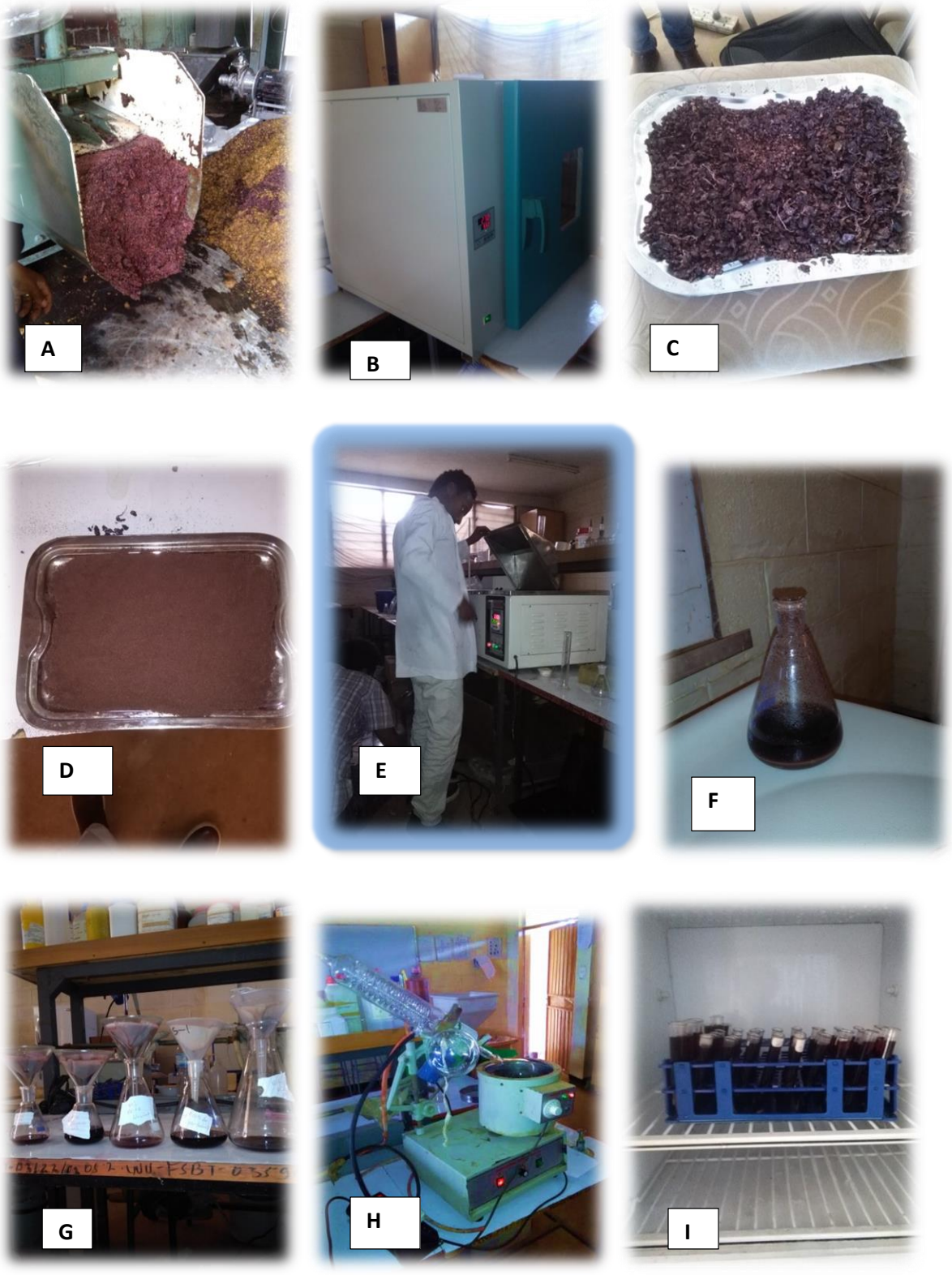


Figure 3.3 General scheme for preparation of grape pomace extracts (from A to I) :(A) Grape pomace; (B, C and D) drying step; (E and F) extraction step under water bath shaker, (G) filtration step,(H) concentration step in a rotary evaporator, (I)grape pomace extracts frozen at-18ºC.

3.3.4 Proximate Composition Analysis of Red Wine and White Wine Grape Pomace

Proximate analysis of Red and White grape pomace such as moisture content, ash content, protein, total carbohydrate, and crude fat were done using the following procedures:

3.3.4.1 Moisture Content

A weighed sample of grape pomace was placed in a ventilated oven where a temperature of 105^{o}C was maintained in accordance with Association of Official Analytical Chemists (AOAC, 2002). Dried sample from the oven was withdrawn and weighed every 3 hours until readings confirmed no significant loss of mass. The moisture content (in percent) was calculated according to equation 3.1.

$$\%moisture = \left[\frac{(m_{initial} - m_{ovendried})}{m_{initial}}\right] * 100\% \quad (3.1)$$

3.3.4.2 Determination of Total Ash

Ash was determined by the method of the Association of Official Analytical Chemists (AOAC, 2002), using the Official Method 923.03. Clean porcelain crucible, dried at 120^{0}C in an oven was ignited at about 550 ^{O}C in a muffle furnace for 3 hours was cooled in desiccators and weighed (M_1). Then 2.0 g samples were weighed into previously dried and weighed (M_2) porcelain crucible. These samples were dried at 120^{0}C for 1 hour and carbonized by oven until the contents turn black. The crucible with the contents were placed in a Muffle furnace (Gallenkamp, Gallenkamp,model SX-2.5-10) set at 550 ^{O}C for 1 hour to ignite until ashing was completed. After this period the crucible with its content was removed and cooled in the desiccators. The crucible with the residue was weighed (M_3). The weights of the ash were expressed as a percentage of the initial weight of the samples. The total ash was expressed as percentages on dry matter basis as follows:

$$Total\ Ash = \left[\frac{M_3 - M_1}{M_2 - M_1}\right] * 100 \quad (3.2)$$

Where: ($M_2 - M_1$) is sample mass in g on dry base and ($M_3 - M_1$) mass of ash in g

3.3.4.3 Determination of Crude Protein

Crude protein was determined by the method of the Association of Official Analytical Chemists (AOAC, 2002) using the Official Method 920.87. Two grams (2.0g) of the samples were weighed into a digestion flask and 0.5g of selenium catalyst was added. 25ml of concentrated H_2SO_4 was added and the flask was shaken to mix the contents. The flask was then placed on a digestion burner for 8h and heated to (370^0C) allowing digestion until the solution turned green and clear. The sample solutions were then transferred into a 100ml volumetric flask and made up to the mark with distilled water. 25ml of 2% boric acid was pipetted into a 250ml conical flask and 2 drops of mixed indicator (20ml of bromocresol green and 4ml of methyl red) solution added, into the decomposition chamber of the distillation apparatus was added 15ml of 40% NaOH solution. 10ml of the digested sample solution was then introduced into a Kjedahl flask. The condenser tip of the distillation apparatus was then dipped into the boric acid contained in the conical flask. The ammonia in the sample solution was then distilled into the boric acid until it completely changed to bluish green. Finally, the distillate was titrated with standardized 0.1N sulphuric acid to a reddish color. The percent total nitrogen and crude protein were calculated using the equation (3.3).

$$Nitrogen(\%) = \frac{(V_2 - V_1) * N * 14.007 * 100}{W_o} \quad (3.3)$$

Where*:*

- V_2 = Volume in ml of standard sulfuric acid solution used in the titration for the test material.
- V_1 = Volume in ml of standard sulfuric acid solution used in the titration for the blank .
- N = Normality of standard sulfuric acid (0.1N).
- W_O = Sample weight on dry matter basis and 14.007 is the molecular weight of nitrogen.

N.B*:* The % of nitrogen is converted to % of protein by using appropriate conversion factor, in this case crude protein content percent per weight = total nitrogen * 6.25 is used.

3.3.4.4 Determination of Crude Fat

Crude fat was determined based on the Sohxlet extraction method of AOAC (2002) using official method 920.39. A 250 ml quick fit round bottom flask was washed and dried in an oven at 105^{o}C for 25 minutes and allowed to cool to room temperature before it was weighed. A clean and dried muslin thimble containing about 5 g of dried sample and covered with fat free cotton at the bottom and top was placed in the extraction chamber. 2.0g of the samples were weighed into the thimble. This was inserted into the extraction column with the condenser connected. 200ml of the extracting solvent (petroleum ether, boiling point 40-60 ^{o}C) was poured into the round bottom flask and fitted into the extraction unit. The flask was then heated with the aid of electro-thermal heater at 60^{o}C for 8 hrs. Losses of solvent due to heating were checked with the aid of the condenser so that it cooled and refluxed the evaporated solvent. After extraction, the thimble was removed and the solvent salvaged by distillation. The flask containing the fat and residual solvent was placed on a water bath to evaporate the solvent followed by a further drying in an oven at 105^{o}C for 30 minutes to completely evaporate the solvent. It was then cooled in desiccators and weighed. The flask containing the extracted fat was dried on a steam bath at 98^{o}C to a constant mass. The fat obtained was expressed as a percentage of the initial weight of the sample using the formula.

$$Crude\ fat, \%by\ weight = \left[\frac{W_2 - W_1}{W}\right] * 100 \qquad (3.4)$$

Where*:* W_1 = weight of the extraction flask (g),

W_2 = weight of the extraction flask plus the dried crude fat (g),

W = weight of samples (g)

3.3.4.5 Determination of Crude Fiber

Crude fiber was determined by the method of the Association of Official Analytical Chemists (AOAC, 2002) using the official method 962.09. About 3.0g defatted samples (from crude fat determination above) were transferred into a 750 ml Erlenmeyer flasks and 200ml of boiling 1.25% H_2SO_4 was added and the flask was immediately set on a hot plate electric oven at 130^{o}C and condenser connected to it. The content was brought to boil within 1 minute and the sample

was digested for 30 minutes. At the end of the 30 minutes, the flask was removed and the content was filtered through a linen cloth in a funnel and subsequently washed with boiling water until the washings were no longer acidic. The samples were washed back into the flask with 200ml boiling 1.25% NaOH solution. The condenser was again connected to the flask and the content of the flask was boiled for 30 minutes. It was then filtered through the linen cloth and thoroughly washed with boiling water until the washings were no longer alkaline. The residue was transferred to a clean crucible with a spatula and the remaining particles washed off with 15ml ethanol into the crucible. The crucible with its content was then dried in an oven at 105^{o}C overnight and cooled in a desiccator and weighed (M_1). The crucible with its content was then ignited in a furnace at 550^{o}C for 2h, cooled and re-weighed (M_2). The loss in weight gave the crude fiber content and was expressed as a percentage of the initial weight of the sample using the formula. The total crude fiber was expressed in percentage as:

$$Total\ Crude\ fiber = \left[\frac{M_1 - M_2}{M_3}\right] * 100 \qquad (3.5)$$

Where*:* M_3 is the weight of samples

3.3.4.6 Determination of Total Carbohydrate

Total percentage carbohydrate was determined by the difference method as reported by Osborne and Voogt (1978). This method involves adding the total values of crude protein, crude fat, crude fiber, moisture and ash constituents of the sample and subtracting it from hundred (100). The value obtained is the percentage carbohydrate constituent of the sample. Total carbohydrate content of the samples including crude fiber was determined by subtraction of the above tested parameters from 100%. Carbohydrate content was determined by difference.

$$Total\%\ C = 100 - [\%M + \%P + \%F + \%Fb + \%A] \qquad (3.6)$$

Where*:* C - Carbohydrate content, M - Moisture content, P - Protein content, F - Fat content, Fb - Fiber content and A - Ash content.

3.4 Characterization of Antioxidants Extract

The best combination of extraction parameters like extraction temperature, time and solvent concentration (ethanol/water ratio) for maximum extract yield was taken for further antioxidant activity evaluation, total phenolic content, total flavonoid, total anthocyanin and preservative effect on sun flower oil were studied.

3.4.1 Yield

Percentage of total yield from each experiment run was calculated using the following formula:
For each factor, total yield is calculated as:

$$Yield(\%) = \frac{g\ extract}{100g\ dried\ grape\ pomace(_{dry\ weight})} \quad (3.7)$$

3.4.2 Color Intensity

The color intensity is determined by the content and structure of the anthocyanins present in a wine grape pomace extracts and is defined as the sum of the absorbances at 420, 520 and 620 nm. Color intensity was calculated as the sum of absorbance at 620, 520, and 420 nm (OIV, 2014).

$$I = A420 + A520 + A620 \quad (3.8)$$

Where: I=color intensity and A=absorbance at 420,520 and 620nm
The hue or tone is conventionally given by:

$$N = \frac{A420}{A520} \quad (3.9)$$

Where: N=hue or tone and A=absorbance

3.4.3 Evaluation of Grape Pomace Antioxidant Activity

The Antioxidant activity of grape pomace extract was determined by UV/ visible light spectrophotometer using DPPH (1, 1-Diphenyl-1-Picrylhydrazyl) free radical scavenging activity.

3.4.3.1 Determination of Free Radical Scavenging Activity of Grape Pomace

The scavenging capacity was read spectrophotometrically by monitoring the decrease in absorbance at 517 nm using a UV-Vis spectrophotometer (Kirby and Schmidt, 2004). This method is based on the reduction of stable DPPH when it accepts hydrogen from an antioxidant compound. Radical scavenging activity of extracts from grape pomace powder against stable DPPH was determined spectrophotometrically. The changes in color (from deep-violet to light-yellow) is measured at 517nm using a UV-visible light spectrophotometer.

Reagents and solutions preparation: deionized or distilled water is used for all recipes and protocol steps. 0.01g of 0.004% DPPH are taken and dissolved in 250ml methanol in volumetric flask. Indeed, 0.075g of standard ascorbic acid was measured and dissolved in 1ml of methanol in 25ml of volumetric flask for comparison. The hydrogen atoms or electrons donation ability of the extracts and some pure compounds was measured from the bleaching of purple colored methanol solution of DPPH. The effect of distilled water extracts on DPPH radical was estimated according to Kirby and Schmidt (2004). Briefly, about 4ml of 0.004% solution of DPPH radical solution in methanol was mixed with 1ml of various concentrations (20, 40, 60, 80, 100 and 120µl) of the extracts in methanol with a vortex mixer. Samples were incubated for 30min in the dark at room temperature. Finally, inhibition of free radical DPPH in percent (%) was calculated in following way:

$$\%\text{Inhibition} = \frac{(A_B - A_A)}{A_B} * 100 \qquad (3.8)$$

Where: A_B absorption of blank sample (t= o min) and

A_A absorption of extract sample (t= 30 min)

Based on the diagram representing the antiradical activity vs. different sample concentrations or the reference compound, the value of IC_{50} was determined. This value represented the sample (or the reference compound) concentration needed for inhibiting 50% of DPPH radicals.

3.4.4 Total Phenolic Content

The total phenol content was determined according to Folin-Ciocalteu's reagent method of modified of Singh and Immanuel (2014). 1ml of Gallic acid or extract solution and 1 ml Folin-Ciocalteu's reagent was mixed and the mixture was incubated at room temperature for 3 min and blue color complex was formed due to redox reaction and lead to increase in absorbance of the extracts. Then 1 ml of sodium carbonate solution was added. The solution was adjusted to 10 ml with distil water or (7ml distil water was added), mixed well and further incubated for 90 min at room temperature and the absorbance was measured at 765 nm using a Visible Spectrophotometer (UV-7804C). Hence, the more rapidly the absorbance increases, the more potent the antioxidant activity of the extract. Gallic acid was used as a positive control. A total phenol value was expressed in terms of Gallic acid equivalent (mg of Gallic acid/g of extracted compound) (Singh and Immanuel, 2014).

The total phenol content was calculated using the following relationship:

$$T = \frac{C * V}{W} \qquad (3.9)$$

Where: C= Gallic acid equivalent concentration obtained from the calibration curve (mg/ml)

V= volume of stock solution of extract (ml)

W = dry weight of extract found in the stock solution (g)

T = total phenol content (mg of GAE/g dry extract)

If dilution of the solution was used for correct concentration to obtain the required absorbance, Dilution factor was not forgotten.

$$T = \frac{C * DF * V}{W} \qquad (3.10)$$

Where: DF = dilution factor

Procedures

Reagent preparation

$NaCO_3$ reagent solution was prepared by weighing 13g of $NaCO_3$& dissolving in 5 ml methanol using 250 ml flask and diluted with distil water; incubate at room temperature for 24 hours. Folin-Ciocalteu's reagent solution was prepared by taking 1ml of Folin-Ciocalteu's into 9ml of distilled water.

Preparation of blank solution

1ml of Folin-Ciocalteu's reagent solution was added to the test tube containing 1ml of methanol, mixed well and incubated for 3 min. After 3 min 1ml of saturated Na_2CO_3 solution was added and adjusted the solution to 10 ml with distil water or (added 7ml distil water), mixed well and incubated in the dark for 90 min at room temperature.

Preparation of standard solution

Gallic acid was used as a positive control or standard for determination of total phenolic content of the extract. A stock solution (50 mg/ml) of Gallic acid was prepared by weighing 2.5g of Gallic acid and dissolving in 5ml methanol and diluted to 50 ml Volumetric Measuring flask using distil water. Aliquots of 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, and 240µL of standard Gallic acid were withdrawn from the stock solution and mixed with 980, 960, 940, 920, 900, 880, 860, 840, 820, 800, 780 and 760µL of methanol solvent to get Gallic acid concentrations of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 mg/ml respectively in separate test tubes. Then 1 ml of Folin-Ciocalteu's reagent was added, mixed well and incubated for 3 min. After 3 min, 1ml of saturated Na_2CO_3 solution was added and adjusted the solution to 10 ml with distil water (added 7ml distil water), mixed well and incubated in the dark for 90 min at room temperature. After 90 min incubation, the absorbance was measured against the blank (the same mixture without the Gallic acid) at 765nm using a UV-Visible Spectrophotometer (UV-7804C).The experiment was carried out in triplicate.

Preparation of sample solution

Sample stock solution of Red wine Grape Pomace Extract (RWGPE) (50 mg/ml) was prepared by dissolving 1g of extract in to 20 ml of ethanol and stock solution of (50 mg/ml) White Wine Grape Pomace Extract (WWGPE) was prepared by dissolving 2g of extract in to 40 ml of ethanol. From each stock solution, 20, 40, 80, 120, 160, 200 and 240µL of sample solution were withdrawn and mixed with 980, 960, 920, 880, 840, 800 and 760µL of methanol to get sample

concentrations of 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 mg/ml respectively in separate test tubes. Then 1 ml of Folin-Ciocalteu's reagent was added, mixed well and incubated for 3 min. After 3 min, 1ml of saturated Na_2CO_3 solution was added and adjusted the solution to 10 ml with distil water (added 7ml distil water), mixed well and incubated in the dark for 90 min at room temperature. After 90 min incubation, the absorbance was measured against the blank (the same mixture without the extract) at 765nm using a UV-Visible Spectrophotometer (UV-7804C). The experiment was carried out in triplicate. The total phenol content was calculated and expressed as milligrams of Gallic acid equivalents (mg of GAE/g dry extract) using the Gallic acid calibration curve and the curve used to determine the corresponding Gallic acid concentration of the samples.

3.4.5 Total Flavonoid Content

The flavonoid content was determined according to aluminum chloride colorimetric method by modification of Singh and Immanuel (2014). Briefly, a dose of 0.25 mL of the extract or catechin standard solution was mixed with 0.75mL of methanol in a test tube, followed by adding 75μL of a 5% $NaNO_2$ solution. After 6 min, 150μL of a 10% $AlCl_3.6H_2O$ (freshly prepared) solution was added and allowed to stand for another 5 min before adding 0.5 mL of 1 M NaOH. The mixture was brought to 2.5 mL with distilled water and mixed well. The absorbance was measured immediately against the blank (the same mixture without the sample) at 510 nm using a UV-Visible Spectrophotometer (UV-7804C). The results were calculated and expressed as milligrams of catechin equivalents (mg of CE/g dry extract) using the calibration curve of catechin. Linearity range of the calibration curve was 10 to 1000 μg/mL (R^2 = 0.99). The non-flavonoid polyphenols was taken as the difference between the total phenol and total flavonoid content (Omoba *et al*., 2015). The total flavonoid content was expressed as milligrams of catechin equivalents (mg of CE/g dry extract) using the calibration curve of catechin. The total flavonoid content was calculated by the following relationship:

$$T = \frac{C * V}{W} \qquad (3.11)$$

Where: C = Catechin equivalent concentration obtained from the calibration curve (mg/ml)
V= volume of stock solution of extract (ml)
W = dry weight of extract found in the stock solution (g)
T = total flavonoid content expressed as (mg of CE/g dry extract)

Procedures

Preparation Reagents

5% $NaNO_2$ solution was prepared by dissolving 5 g of $NaNO_2$ in to 100 ml of distill water and 10% $AlCl_3.6H_2O$ was prepared by placing 10 mg $AlCl_3$ in to 100 ml of distill water. Similarly, 1 M NaOH was prepared by adding 0.4 g of NaOH in to 10 ml of distill water.

Preparation of blank solution: the test tube containing 1ml of methanol, 75μL of a 5% $NaNO_2$ solution was added. After 6 min, 150µl of a 10% $AlCl_3.6H_2O$ was added followed by addition 1 M NaOH after another 5 min stand and the mixture was brought to 2.5 mL with distilled water and mixed well.

Preparation of standard solution: A stock solution (0.5 mg/ml) of catechin was prepared by dissolving 0.05 g of catechin in to 100 ml of methanol. Aliquots were withdrawn from the stock solution to get catechin concentrations 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 mg/ml by mixing 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml of catechin stock solution with 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 and 0 ml of methanol respectively in the different test tubes. 75μL of a 5% $NaNO_2$ solution was added in each test tube. After 6 min, 150µl of a 10% $AlCl_3.6H_2O$ was added to each test tube. Again after another 5 min, 0.5 mL of 1 M NaOH was added and the mixture was brought to 2.5 mL with distilled water and mixed well. The absorbance was measured immediately against the blank (the same mixture without the catechin) at 510 nm using a UV-Visible Spectrophotometer (UV-7804C). The experiment was carried out in triplicate. The standard catechin calibration curve was developed by plotting linear regression curve of absorbance versus catechin concentration (mg/ml).

Preparation of sample solution: Sample stock solution of RWGPE (4 mg/ml) was prepared by dissolving 0.1702g of extract in to 43 ml of ethanol and stock solution of WWGPE (3.5 mg/ml) was prepared by dissolving 0.086 g of extract in to 143.33 ml of ethanol. From each stock solution, 0.25 ml was withdrawn from the stock solution and mixed with 0.75 ml of methanol to get a sample concentration of 1 mg/ml for RWGPE and 0.5 mg /ml for WWGPE in the distinct test tube. 75μL of a 5% $NaNO_2$ solution was added in each test tube. After 6 min, 150µl of a 10% $AlCl_3.6H_2O$ was added to each test tube. Again after another 5 min, 0.5 mL of 1 M NaOH was added and the mixture was brought to 2.5 mL with distilled water and mixed well. The absorbance was measured immediately against the blank (the same mixture without the catechin) at 510 nm using a UV-Visible Spectrophotometer (UV-7804C). The experiment was carried out in triplicate.

3.4.6 Determination of Total Anthocyanins Concentration

The total Anthocyanins concentration was determined by using the pH differential method which was proposed by Rockenbach *et al*.(2012).They propose the equation:

$$A = (Abs_{\ 510\ nm} - Abs_{\ 700\ nm})pH1.0 - (Abs_{\ 510\ nm} - Abs_{\ 700nm})pH4.5 \quad (3.12)$$

1gm from the extracts was taken and dissolved in 10ml of acidified methanol (85ml methanol + 15ml 1% HCl), then 0.8ml from this mixture were taken and the volume was completed to 3ml with potassium chloride (0.025M, pH 1.0), the same steps were repeated by dilution with 3ml from Sodium acetate buffer(0.4M,pH 4.5). The absorbencies of the two dilutions of the sample were taken at maximum wave length and 700nm, these dilutions were left equilibrate for 15min. (Absorbance readings were made against water blank). The same procedure was repeated by using acidified ethanol and water (Rockenbach *et al*.,2012). The total anthocyanin concentration in the original sample is calculated using the following equation:

$$\text{Total anthocyanin}\left(\frac{\text{mg}}{\text{L}}\right) = \frac{(A \times \text{MW} \times \text{DF} \times 1{,}000)}{(e \times L)} \quad (3.13)$$

Where:

- MW = 449.2, the molecular weight of Cyanidin 3- *O* -glucoside chloride (Cyd-3-glu);
- DF= dilution factor;
- *e* = 26,900, the molar absorptivity of Cyd-3-glu;
- *L* = 1 cm, the path length of cuvette.

3.4.7 Preservative Effect of Grape Pomace Extracts on Soybean Oil

Inhibition of oil oxidation was investigated as potential food applications of the extracts. As it concerns prevention of oil oxidation, some preliminary tests were carried out according to the *Shaal Oven Test* (or accelerated resistance test) which simply consists in monitoring the increase of peroxides value (PV) in an oil sample kept in a thermostatic oven at 65°C.

The addition of antioxidants should avoid or, at least, delay, the peroxides formation. Soybean oil was bought at a local market. As suggested by Bandonienė *et al*. (2000), refined soy oil, free of additives, was used as the substrate for oxidation studies. Soy bean oil samples (100mL) containing 400, 800, 1200 and 1600 ppm or (0.4, 0.8, 1.2 and 1.6 g) RWGPE and WWGPE extract (ethanol) were separately prepared and placed in 250ml glass beakers. Synthetic

antioxidants Butylated hydroxytoluene (BHT) taken from Food Science and Nutrition Department, Addis Ababa University were mixed in soy bean oil for a comparative study at their legal limit of 200 ppm(0.2g). Control samples without antioxidant were also placed under identical conditions. Each beaker was covered with aluminum foil, placed into an electric oven and subjected to accelerated oxidation at 65^{o}C for 7 days. All oil samples of each treatment were prepared in triplicate. Oil samples were withdrawn every 24hr to assess PV (peroxide value) and measurement was done every one day for seven consecutive days according to the AOCS method Cd 23-93 (Duh and Yen., 1997).

3.4.7.1 Determination of Peroxide Value

The Peroxide value is evaluated according to AOCS Official Method Cd 8-53 (2002).
Five grams oil samples were weighed into a conical flask and 30 ml of solvent mixture of glacial acetic acid-chloroform in the ratio of 3:2, respectively, was added to the oil samples. Half ml saturated potassium iodide (KI) solution was added to the solution and allowed to stand for 1 min. Thereafter, 30 ml of distilled water is added and titrated with 0.01 N sodium thiosulfate solution using starch indicator until the yellow color is discharged. A blank was prepared alongside the oil samples. Peroxide value is calculated as:

$$PV = \frac{10 * (V_1 - V_2)}{m} \qquad (3.14)$$

Where*:* V_1 =volume of $Na_2S_2O_3$ for determination of test samples in ml, V_2 =volume of $Na_2S_2O_3$ for determination of blank solution in ml and m = mass of test portion in g (5g)

3.5 Experimental Design and Statistical Analysis

In this study, the Face Centered Central Composite Design (FCCCD) under the Response Surface Methodology(RSM) was used to determine the influence of extraction solvent concentration(ethanol to water ratio), extraction temperature and extraction time on the total yield of Red and White grape pomace antioxidants extracts and to identify the optimum levels because of Response Surface Methodology (RSM) is a “collection of mathematical and statistical

techniques used for modeling and analyzing problems in which a response of interest (output) is influenced by several variables (input)" (Montgomery, 2005).

The effective use of RSM design can result in reduced variability and closer conformance to the target optimization parameters. Other advantages of RSM compared to the one variable input model include the accumulation of large amounts of information from small numbers of samples, understanding potential interactions of input variables on the response through the construction of contour maps, and determining a model equation (Bas and Boyac, 2007).The three independent variables (solvent concentration, temperature and time) were investigated at three levels as shown in Table 3.1. A 2k factorial design gave a total of 17 experimental runs.

$$\textbf{Runs} = 2^k + 2k + n_c = 2^3 + 2(3) + 3 = 8 + 6 + 3 = 17$$

Where: k is the factors and n_c is center point.

A three-factor and three level face center central composite design consisting of 17 runs for total yield were employed including three replicates at the center point (Table 3.1). The experimental design is presented in Table 3.2. Experimental design was analyzed and done by Design-Expert 7.0.0 software. The quality of fit of the regression model expressed as the coefficients of determination (R^2), the statistical significance determined by ANOVA the response surface and the contour plots were all study to estimate the models as well as to determine the optimum levels. For each factor, an experimental range is adjusted based on the results of literature data and on the performance of preliminary experiment trials.

Table 3.1 Levels of independent variables for extraction process based on central composite design (CCD)

Independent variable	**Units**	**Factor**	**Coded Levels**		
			-1	**0**	**+1**
Solvent ratio	(v:v)	X1	60:40	75:25	90:10
Time	minutes	X2	60	90	120
Temperature	^oc	X3	30	45	60

Table 3.2 Three factors, three- level face-centered cube design with 3 center points used for RSM, (Coded and uncoded) parameters

Standard Order	Factor X1	Factor X2	Factor X3	Solvent Ratio (v:v)	Time (minutes)	Temperature (^{o}c)
1	-1.00	1.00	-1.00	60:40	120	30
2	0.00	-1.00	0.00	75:25	60	45
3	1.00	1.00	1.00	90:10	120	60
4	1.00	0.00	0.00	90:10	90	45
5	1.00	1.00	-1.00	90:10	120	30
6	-1.00	1.00	1.00	60:40	120	60
7	0.00	0.00	0.00	75:25	90	45
8	-1.00	0.00	0.00	60:40	90	45
9	1.00	-1.00	1.00	90:10	60	60
10	-1.00	-1.00	1.00	60:40	90	60
11	-1.00	-1.00	-1.00	60:40	60	30
12	0.00	0.00	0.00	75:25	90	45
13	1.00	-1.00	-1.00	90:10	90	45
14	0.00	0.00	0.00	75:25	90	45
15	0.00	0.00	1.00	75:25	90	60
16	0.00	1.00	0.00	75:25	120	45
17	0.00	0.00	-1.00	75:25	90	30

Chapter Four

Results and Discussion

4.1 Proximate Composition of Red and White Wine Grape Pomaces

The moisture contained in Red grape pomace was higher than the moisture contained in White Grape Pomace (see Table 4.1). As for the Grape Pomace (GP) powder obtained upon oven drying, its moisture content is below 5%. The major components identified in both residues GP powder from red wine making (RWGP) and white wine making (WWGP) were moisture content, crude protein, ash, crude fat, crude fiber and carbohydrates. Results from respective analyses were used to characterize red wine grape pomace and white wine grape pomace as presented in Table 4.1.

Table 4.1 Composition of Red and White grape pomaces powder in % dry basis

Component (% dry basis)	Results (mean ± SD)	
	Red grape pomace(RWGP)	White grape pomace (WWGP)
Moisture (g/100g)	3.33± 0.5	3.25 ±0.8
Total Ash (g/100g)	4.20 ±0.4	4.59±0.1
Crude Protein (g/100g)	13.30 ±1.2	10.14±0.7
Carbohydrate (g/100g)	28.0 ±0.4	31.83±2.4
Crude fiber(g/100g)	44.17 ±0.7	43.86±0.3
Crude fat(g/100g)	7.16 ±1.0	6.33±0.5

The results showed that red grape pomace contained higher moisture content (3.33%), Crude Protein (13.3%), crude fat (7.16%) and crude fiber (44.17%) compared to white grape pomace. The pomace of the white grape pomace showed a higher content in Total ash (4.59%) and carbohydrate (31.83%) compared to red grape pomace. These results were compared with corresponding values given in literatures and proved that they are in the same range as Ethiopian wine grape pomaces for both white and red wine grape pomaces. The study carried out by

Mendes *et al*.(2013) on the chemical composition of the grape pomace from five varieties of grapes (two white and three red) revealed that white grapes have a protein content of 5.38 - 6.54% and red ones consists of 11.26 to 12.34% protein. The content differences may be due to the analyzed grape varieties, fertilization conditions and the soil type. Regarding the content of Crude carbohydrate, Bravo (1998) obtained values ranging between 17.3 to 29.0% for both the white and red grapes pomace which is very similar to the values obtained in this research (31.83%) and (28.0%), respectively.

4.2 Effect of Extraction Parameters on Red and White Grape Pomace Yield

The yield of extracts obtained from red wine grape pomace (RWGP) and white wine grape pomace (WWGP) at each of the17 run is given in Table 4.2.

Table 4.2 Extracts yield at different processing conditions for Red and White grape pomaces

Standard Order	Solvent Ratio(v:v)	Time (min.)	Temperature (^{o}c)	Yield (%)	
				Red grape pomace	White grape pomace
1	-1.00	1.00	-1.00	36.56	25.43
2	0.00	-1.00	0.00	37.13	29.23
3	1.00	1.00	1.00	52.35	48.56
4	1.00	0.00	0.00	50.32	40.23
5	1.00	1.00	-1.00	42.06	35.45
6	-1.00	1.00	1.00	38.33	29.86
7	0.00	0.00	0.00	48.03	41.20
8	-1.00	0.00	0.00	38.23	30.02
9	1.00	-1.00	1.00	48.65	41.35
10	-1.00	-1.00	1.00	33.13	22.69
11	-1.00	-1.00	-1.00	30.23	20.97
12	0.00	0.00	0.00	42.03	31.52
13	1.00	-1.00	-1.00	45.51	37.21
14	0.00	0.00	0.00	42.62	35.85
15	0.00	0.00	1.00	48.51	42.05
16	0.00	1.00	0.00	45.18	41.89
17	0.00	0.00	-1.00	40.12	30.78

It can be seen from Table 4.2 that the better extraction yield was obtained with Red Wine Grape pomace (RWGP) at run number (3) and also at the same run for WWGP. Data for percentage yield were generated as per equation 3.7 given in section 3.5.1. Percentage yield varied within the range of 30.23% to 52.35% for RWGP and 20.97% and 48.56% for WWGP respectively form minimum to maximum yield according to this result RWGP gives higher yield than WWGP, this may be due to the levels of anthocyanins in RWGP grapes is higher than WWGP that gives red color to red wine . The minimum percentage yields were at 60:40 solvent ratio, 30 ^{o}c temperature and 60 minutes at run number (11) which was observed at lower ethanol ratio, temperature and time and the maximum percentage yield were at 90:10 solvent ratio, 60^{o}C temperature and 120 minutes at run number (3) (Table 4.2), maximum yield was observed at higher ethanol concentration, temperature and time for both RWGP and WWGP.

As can be seen from Table 4.2 as temperature increases from low level coded (-1) to higher level coded (+1) extraction yield increases because temperature is one of the most important variables to affect the release of the phenolic compounds of the wine grape pomaces; increases in the temperature of extraction contribute to improve both the solubility of the solute and the diffusion coefficient, consequently, in high temperatures, there is an increase in the content of extracted compounds (Pinelo *et al*., 2005) and other studies have shown that elevated temperatures result in higher extract yield (Liyana-Pathirana *et al*., 2006) and heating favors the process of extraction of winery by-products, since it increases the solute diffusion ratio, accelerating the transference of mass, solubilizing compounds, and reducing solute–matrix interactions (Mendes *et al*., 2013) .

Temperature was influent on extracts yields for both RWGP and WWGP, because temperature increase favored extraction by increasing solubility and diffusion coefficient of any compounds, not only of antioxidants (Pinelo *et al*., 2005) which is in agrement to this study. Similarly for solvent concentration the performance of this system can be explained with a dual mixture in this research, particularly a mixture of an organic solvent (ethanol) and water, the extraction efficiency is improved as ethanol ratio increases from lower level coded (-1) to higher level coded (+1) this was due to the organic solvent enhances the solubility of the analyte and also water increases the analyte desorption (Mendes *et al*., 2013).And also found out that extracts made of both red and white wine grape pomace, the yield of extract gently increased with the time.

As time increase from lower level (-1) to higher level coded (+1) the response yield increases which is similar result found out by the work of Lapornik *et al*.(2005) reported extract yield strongly increased with longer time of extraction similar in both grape pomaces. Intermediate and higher values were reached in agreement with other literature works Pekić *et al*. (1998) and solvent concentration, time and temperature combinations gave actually the same results, in case of investigating the influence of addition of water to ethanol, since mixtures of alcohols and water have revealed to be more efficient in extracting phenolic constituents than the corresponding mono-component solvent system (Pinelo *et al*., 2005).

Regarding grape and wine-making wastes, influence of water content of alcohols has been studied only for extraction from seeds (Alonso *et al*., 1991). In fact, ethanol, a polar solvent, effectively extracts flavonoids and their glycosides, catecols and tannins from raw plant materials (Bazykina *et al*., 2002), but solubility of these compounds can be enhanced using a mixed solvent over a limited compositional range (Cacace and Mazza, 2003).

Maximum extract yields were obtained at decreasing water content of ethanol as shown in Table 4.2 in both RWGP and WWGP. Yield increases with the increase in solvent ratio or ethanol concentration, time and temperature, as expected, from around 30.23% at 30 ºC to around 52.35 % at 60 ºC in RWGP and from 20.97 % to 48.56% in WWGP, With the increase in temperature, the ionic product of water increases, and water becomes a stronger catalyst for the hydrolysis of biomass. Carbohydrates are the major component of WGP (Table 4.1), comparing the raw material WWGP with RWGP, the lower yield obtained with WWGP (48.56%) compared to RWGP (52.23%), this can be due to the higher amount of carbohydrates present in this residue, which is higher in the amount of carbohydrates in WWGP which contain some insoluble sugar which bounded to phenolic compound and prevent extraction (Pekić *et al*., 1998).

In the perturbation plot, Figure 4.1 and Figure 4.2 for RWGPE and WWGPE respectively shows the effects when all factors at the center point in the design space are compared. The perturbation plot assists in comparison of the effects of all factors at a particular point in the design space; when the factor curvature is sharper, the factor effect is more important to the response. The plot was obtained for 75:25 solvent ratio,90 minute of mix time and 45ºC temperature, Figure 4.1

and 4.2 shows that the response of the extracts yield of RWGP was very sensitive to solvent ratio, followed by the temperature and finally by mix time.

The extract yield of WWGP was very sensitive to solvent ratio, followed by temperature and mix time but in this case mix time shows close importance to temperature. As it is observed from this graph, as each parameter increases and passes the reference point the yield also increases, especially, the yield is highly increased with the increasing of extraction solvent ratio from lower level to higher level and temperature for RWGP but start decreasing as mix time increases further but in case of WWGP it become increases in yield with increasing of solvent ratio, time and temperature. This is because high extraction temperature improves extraction efficiencies due to heat renders the cell walls more permeable to solvents and components and increases the solubility and diffusion coefficients of the components to be extracted.

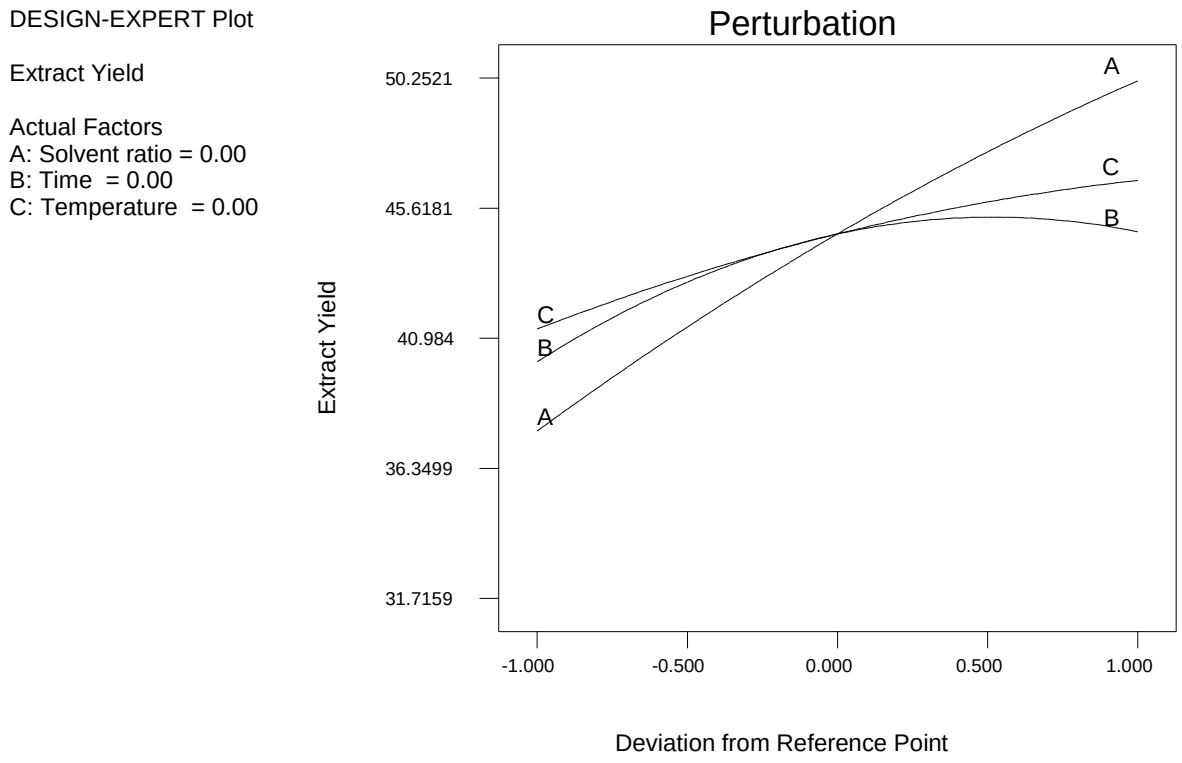


Figure 4.1 Perturbation graphs showing the interaction of factors for Red Wine Grape Pomaces

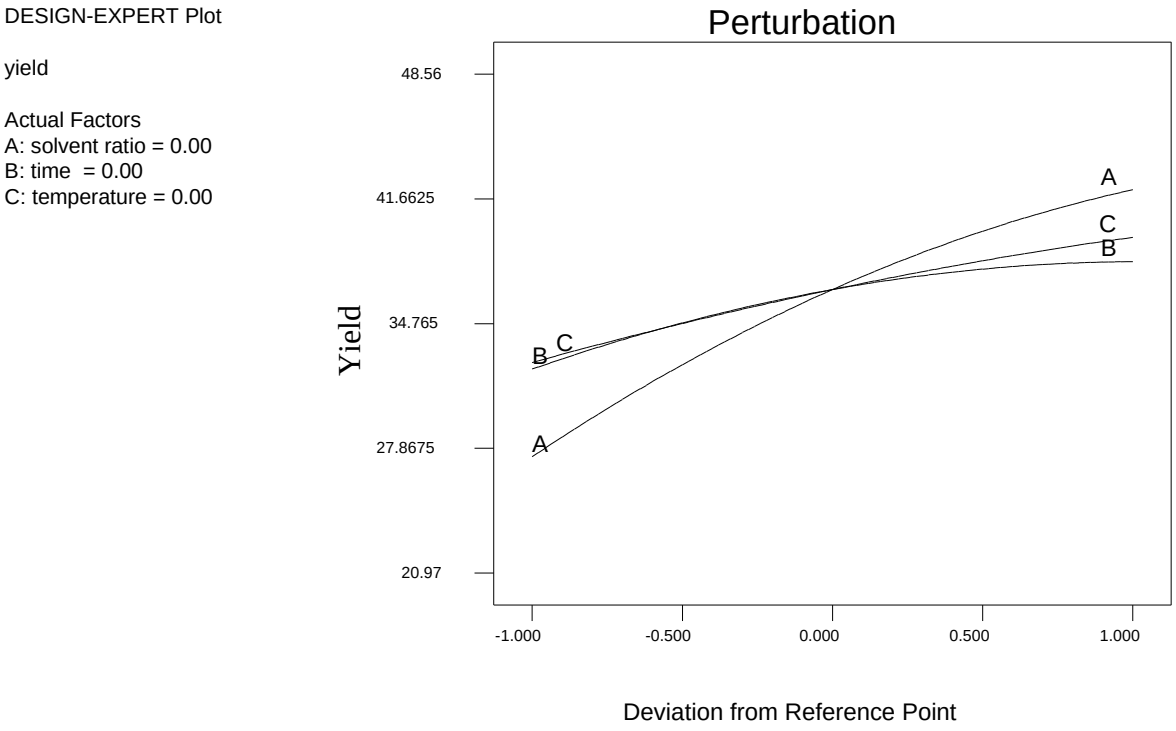


Figure 4.2 Perturbation graphs showing the interaction of factors for White Grape Pomace

4.1.1 Development of Regression Model Equation

Table 4.2 summarizes the result obtained with the experimental design which was aimed in determining the conditions that favors maximum yield increase in RWGP and WWGP extracts. A quadratic model equation 4.1 and 4.2 for RWGP and equation 4.3 and 4.4 for WWGP in terms of coded factor and actual factor shown below were fitted to the data model for predicting response; yield of extracts, respectively. Using the experimental results for extract yield as shown in Table 4.1, response surface model was developed for the adequacy of the model is then performed in the subsequent step. The F ratio is calculated for 95% level of confidence. The final response equation for extract yield is given by quadratic model as equation 4.1 and 4.3 for RWGPE and WWGPE based on coded factors and equation 4.2 and 4.4 based on actual factors, respectively.

For Red Wine Grape Pomace Extracts (RWGPE)

Final equation in terms of coded factors: Red wine grape pomace extracts (RWGPE):

Yield = +44.70 +6.24* A +2.32* B +2.65* C -0.78* A^2 -2.24* B^2 -0.74* C^2 -1.41 * A * B
+1.10 * A *C +0.75* B * C (4.1)

Where: A = solvent ratio
B = time
C = temperature

Final Equation in Terms of Actual Factors for RWGPE:

Yield =

+44.70085+6.24100* Solventratio +2.31600* Time +2.64900 *Temp - 0.78148*Solvent ratio^2-2.23648*Time^2-0.74148*Temp^2-1.41000*Solventratio*Time+1.09500*Solventratio*Temp
+0.75250 * Time * Temp (4.2)

As shown in the final equation in terms of coded factors, the response yield was affected by both linear terms A, B, C and quadratic terms, pure quadratic terms (A^2, B^2, and C^2) and interaction quadratic terms (AB, AC, BC). All coefficients of linear terms were positive and the response yield was positively affected by linear terms but the coefficients of interaction terms were negative and the response yield was negatively affected by quadratic terms. From the linear effects, solvent ratio concentration had highest effect on response yield. Similarly, pure quadratic term of extraction time (B^2) had highest effect on response yield from negative quadratic effects. On the basis of the coefficients in equation 4.1 for RWGP, it was evident that the percentage of extract yield increases with the solvent ratio (A) and extraction time (B) and temperature (C). Solvent ratio concentration has a more profound effect on extract yield as compared to extraction time. As expressed in equation 4.1, solvent ratio, extraction time and temperature showed significantly linear effects on the RWGPE (Red Wine Grape Pomace Extract) yield, which was clearly indicated by the largest positive linear regression coefficient 6.24, 2.32 and 2.65, respectively.

White wine grape pomace extracts (WWGPE):

Final equation in terms of coded factors: white wine grape pomace extracts (WWGPE):

Yield = +36.65 +7.38 * A +2.97 * B +3.47 * C -1.87 * A^2 -1.43 * B^2 -0.58 * C^2

- 0.77* A * B +1.39* A * C +1.46 * B * C (4.3)

Where: A = solvent ratio

B = time

C = temperature

Final Equation in Terms of Actual Factors for WWGPE:

Yield =

+36.64746+7.38300*solventratio+2.97400* time +3.46700*temp-1.86556*solventratio^2

1.43056* time ^{2} -0.57556* temp^2-0.77250* solvent ratio * time +1.38750 * solvent ratio * temp

+1.46000* time * temp (4.4)

For WWGPE it shows similar as RWGPE , WWGPE on the basis of the coefficients in equations 4.3 it was evident that the percentage of extract yield increases with the solvent ratio (A) and extraction time (B) and temperature (C). Solvent ratio concentration has a more profound effect on extract yield as compared to extraction time. As expressed in equation 4.3, solvent ratio, extraction time and temperature showed significantly linear effects on the WWGPE (White Wine Grape Pomace Extract) yield, which was clearly indicated by the largest positive linear regression coefficient 7.38, 2.97 and 3.47 (A,B and C) respectively. As shown in the final equation in terms of coded factors, the response yield was affected by both linear terms (A, B, C) and quadratic terms, pure quadratic terms (A^2, B^2, and C^2) and interaction terms (AB, AC, BC). All coefficients of linear terms were positive and the response yield was positively affected by linear terms but the coefficients of interaction terms for AB is negative but AC and BC were affect positively and also the response yield was negatively affected by quadratic terms. From the linear effects, solvent ratio concentration had highest effect on response yield. Similarly, pure

quadratic term of extraction time (B^2) had highest effect on response yield from negative quadratic effects.

Generally the positive or negative impact of many variables such as solvent ratio, time contact, temperature and solvent-to-solid ratio etc., on the mass transfer of the extraction process is not always obvious. Each system consisting of a material-solvent combination shows an unpredicted different behavior, probably due to the solvents chemical characteristics and the various composition and structure of the natural products (Hollmann and Katan, 1999). Statically interpretation shows the threshold of significance at 95% obtained after using the ANOVA test for the analysis of the coefficients of regression models, they are statistically considered as significant. The reported positive and negative effects of the studied parameters on the extract yield for RWGPE and WWGPE were expected, despite the significant positive effect of temperature elevation on the extraction yield, its augmentation should be limited; the membranes denaturation and phenolic compounds stability are threatened above 60°C (Kim *et al*., 2006). Moreover, subjecting grape pomace to high temperatures might liberate certain phenolic compounds while concurrently promoting possible thermal decomposition of others, which were already released at lower temperatures (Wijngaard *et al*., 2012).

4.1.1.1 Model Adequacy Checking of Extraction Yield for Red and White Wine Grape Pomaces

It is always necessary to examine the fitted model to ensure that it provides an adequate approximation to the true system and verifies that none of the least squares regression assumptions are violated. In general, ensuring the model exhibits a good fit with experimental data is essential to avoid poor or misleading results (Baş and Boyaci, 2007). Therefore, the adequacy of the model was evaluated by applying the quality of the model developed; it could be evaluated from their coefficients of correlation.The closeness to one of the R^2 >75% values for RWGPE and WWGPE indicates a high degree of correlation between the observed and predicted values, which means that a reasonable agreement of the corresponding models with the experimental results is found (Baş and Boyaci, 2007). Using ANOVA table 4.3 and table 4.4 for RWGPE and WWGPE, respectively shown below, the coefficients of regression analysis was carried out, enabling the determination of the Lack of fit significance of each extraction model ,avoiding hence poor and misleading results (Montgomery, 2005). The coefficient of variance

(CV) as the ratio of the standard error of estimate to the mean value of the observed response (as a percentage) is a measure of reproducibility of the model and as a general rule a model can be considered reasonably reproducible if its CV is not greater than 10% (Pinelo *et al*., 2005).In this case the CV of RWGPE is 7.25% and for WWGPE 9.33% so the model for both RWGPE and WWGPE were reasonably reproducible.

The regression model was found to be significant with the correlation coefficients of determination of R-Squared, adjusted R-Squared and predicted R-Squared of RWGPE and WWGPE having a value of 0.8981, 0.7670, 0.1972 and 0.8871,0.7819,0.3374, respectively.

The values of R-squared for RWGPE and WWGPE for the developed correlation are 0.8981 and 0.8871; it implies that 89.71% and 88.71% of the total variation in the yield of extract are attributed to the experimental variables studied. In the other word, only 10.29% RWGPE and 11.29 % WWGPE of the total variation were not explained by the model.

The "Adeq Precision" measures the signal to disturbance ratio due to random error. A ratio greater than 4 is desirable. Here ratio of RWGPE and WWGPE 9.483 and 9.255 respectively indicates an adequate signal. Therefore, this model can be used to navigate the design space.

The normal probability plots of residuals were shown in Figure 4.3 for, RWGPE and Figure 4.6 for WWGPE. It is expected that data from experiments form a normal distribution it reveals that the residual fall on a straight line, implying that the errors are spread in a normal distribution. Here a residual means difference in the observed value (obtained from the experiment) and the predicted value or fitted value. This is also, confirmed by the variations between the experimental results and model predicted values analyzed through residual graphs, and are presented in Figure 4.4 and Figure 4.7 for RWGPE and WWGPE, respectively. On the other hand, Figure 4.5 and 4.8 shows the plot of externally studentized residuals versus predicted values for RWGPE and WWGPE, since the points are in random and show no pattern, the model is suitable to the data. It also satisfies the independent normally distributed residuals that are usually assumed (Montgomery, 2005). Figure 4.4 and 4.7 that is residual versus predicted plot and Figure 4.5 and 4.8 that is predicted versus actual plot for both RWGPE and WWGPE shows how precisely the antioxidants extract yield is modeled, because all the points line up well and the deviation of points for extract yield from normality is insignificant. In addition, the normal probability plot indicates the residuals following a normal distribution, in the case of this

experiment the points in the plots shows fit to a straight line in the figure, this shows that the quadratic polynomial model satisfies the assumptions analysis of variance (ANOVA) *i.e.* the error distribution is approximately normal. The graph of the predicted values obtained using the developed correlation versus actual values is shown in Figure 4.5 and 4.8. The outcomes in Figure 4.5 and 4.8 demonstrated that the regression model equation provided a very accurate description of the experimental data, in which all the points are very close to the line of perfect fit. This result indicates that it was successful in capturing the correlation between the three extraction process variables to the yield of extract in both RWGPE and WWGPE. The adequacy of the model was further checked with analysis of variance (ANOVA) as shown in table 4.3 and table 4.4 for RWGPE and WWGPE, respectively. Based on a 95% confidence level, F–value is a test for comparing model variance with residual (error) variance. If the variances are close to the same, the ratio will be close to one and it is likely that any of the factors have a significant effect on the response with the P–value less than 0.0500. It is calculated by model mean square divided by residual mean square.

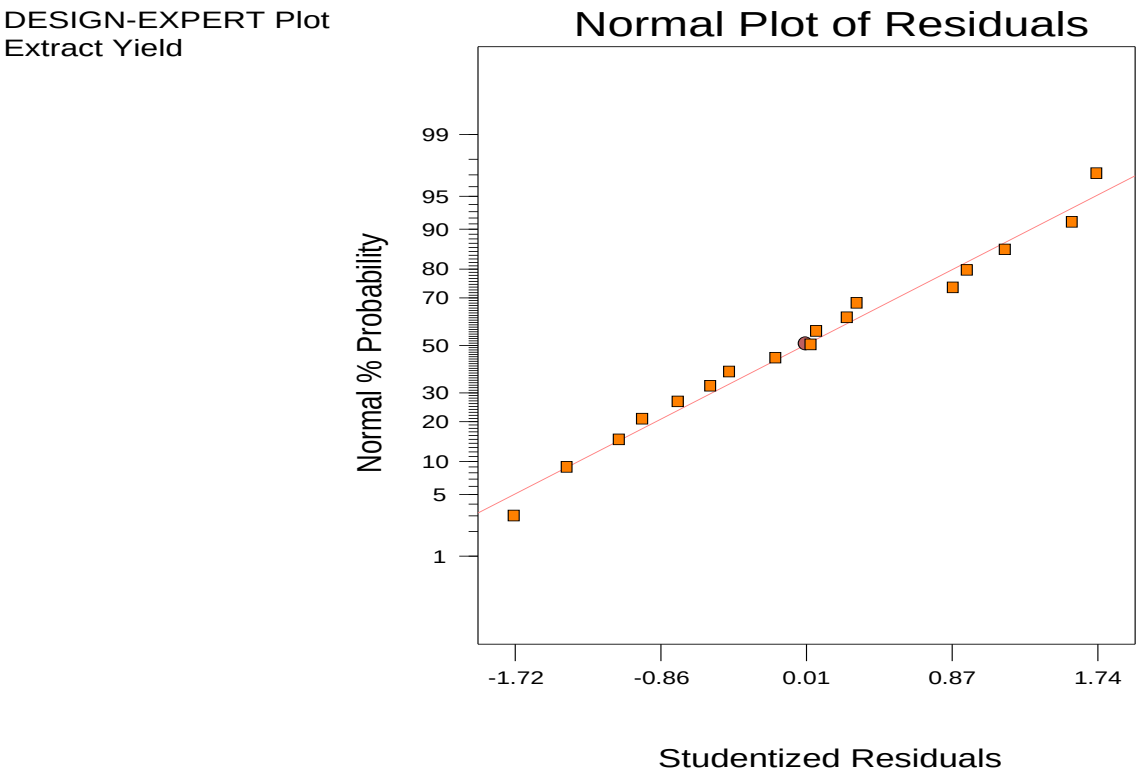


Figure 4.3 Normal plots of residuals yield of Red Wine Grape Pomace Extracts

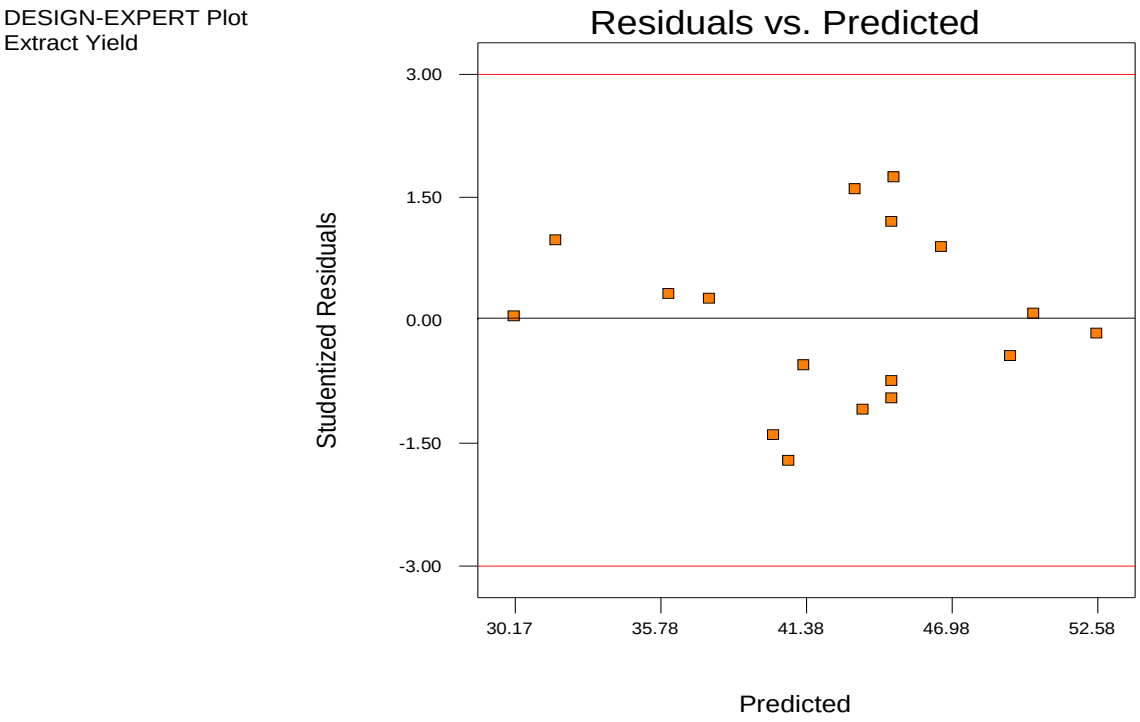


Figure 4.4 Residual versus predicted plot yield of Red Wine Grape Pomace Extracts

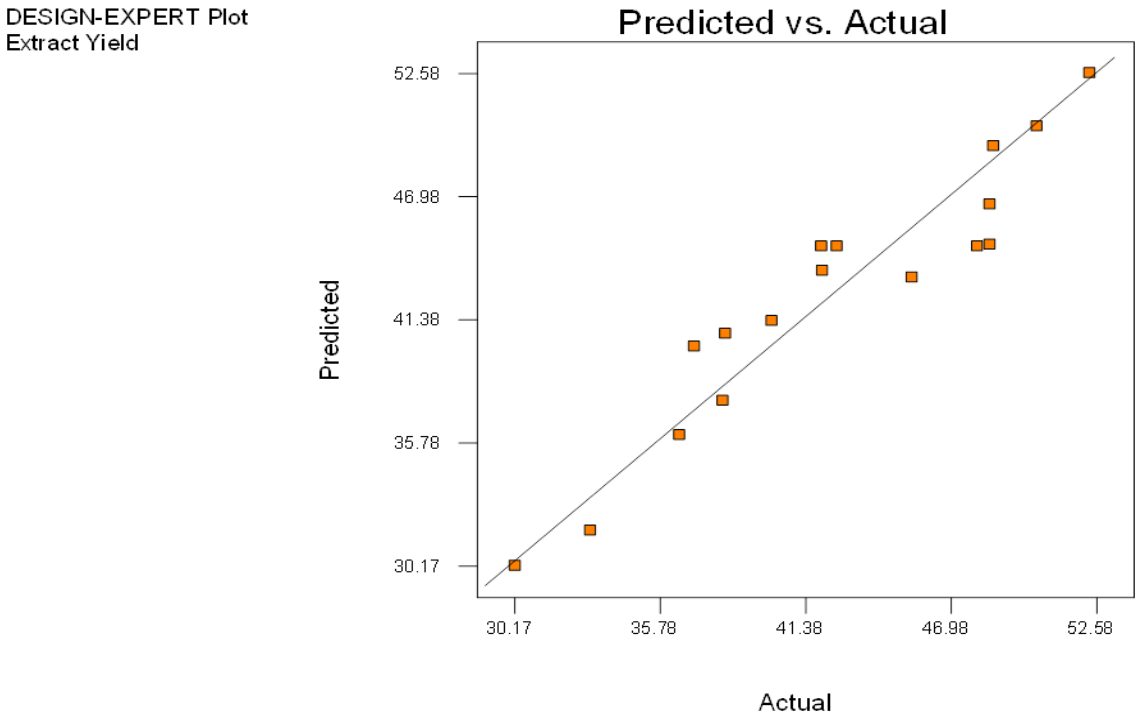


Figure 4.5 Predicted versus Actual plot yield of Red Wine Grape Pomace Extracts

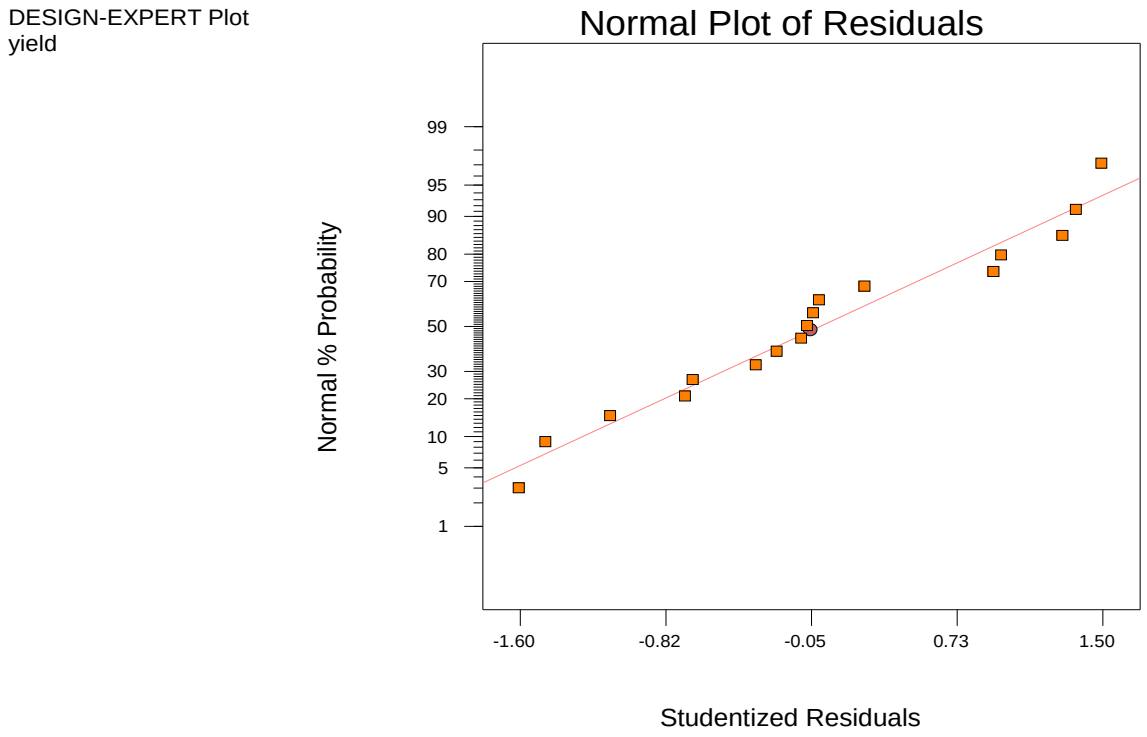


Figure 4.6 Normal plots of residuals yield of White Wine Grape Pomace Extracts

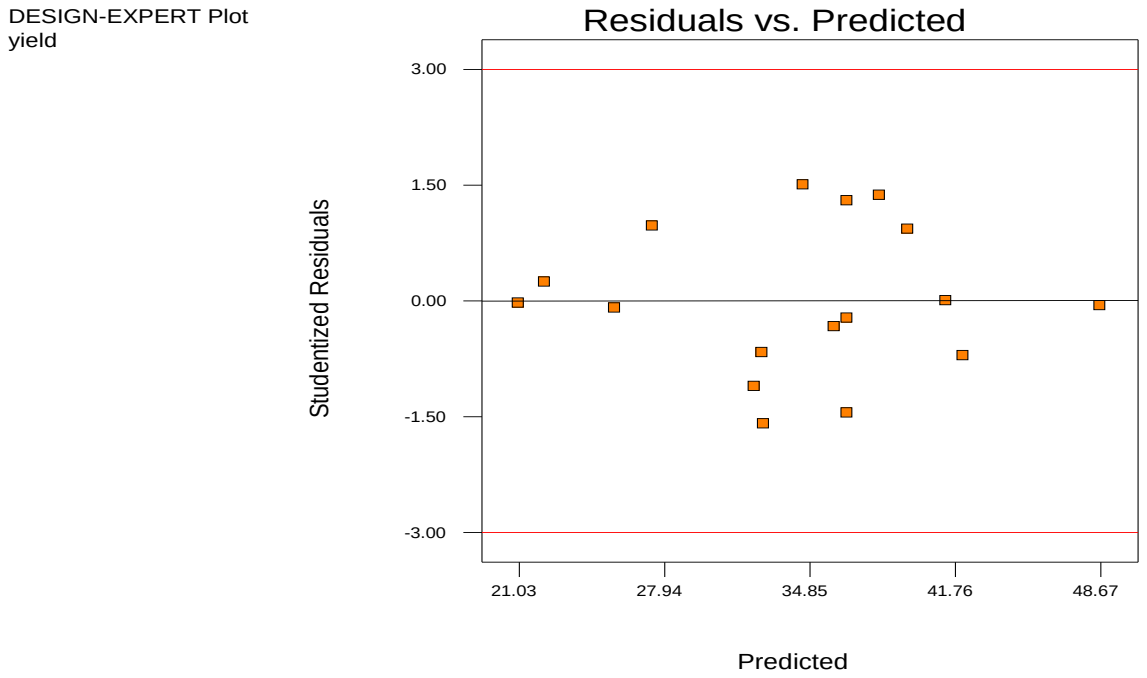


Figure 4.7 Residual versus predicted plot yield of White Wine Grape Pomace Extracts

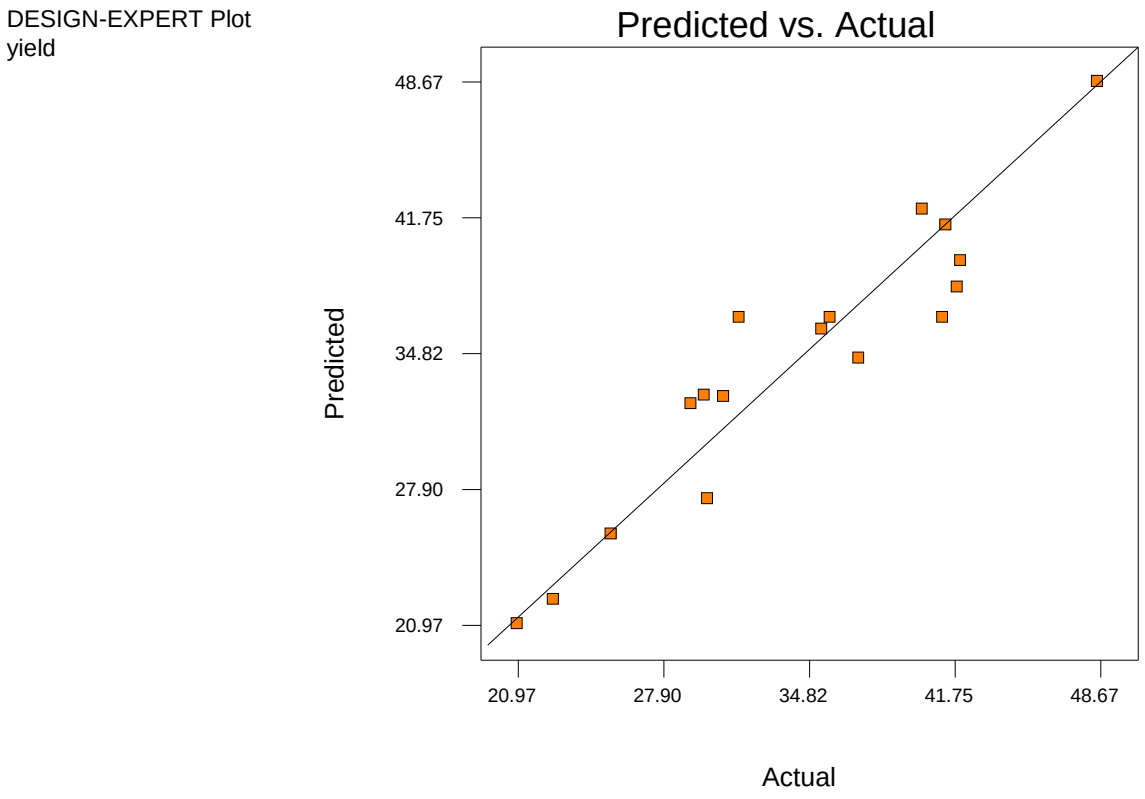


Figure 4.8 Predicted versus Actual yield of White Wine Grape Pomace Extracts

Table 4.3 and Table 4.4 for RWGPE and WWGPE, respectively shows the values of 'P' for each term on the yield of extract , the value of 'P' less than 0.05 (i.e., α=0.05, or 95% confidence) indicates that the obtained models are statistically significant for both RWGPE and WWGPE as depicted in table 4.3 and table 4.4 form ANOVA analysis. The solvent ratio, time and temperature are found to be significant factors that affect the yield of extract.

Whereas the interaction effect of the input variables (AB), (AC) and (BC) as well as pure quadratic term A^2, B^2, and C^2 are insignificant in both red wine grape pomace extracts and white wine grape pomace extracts(see Table 4.3) for RWGPE yield and for WWGPE(see Table 4.4) below for ANOVA analysis.

Table 4.3 Analysis of Variance (ANOVA) for the regression model equation and coefficients of RWGPE

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	Model Significance
Model	585.59	9	65.07	6.85	0.0095	significant
A	389.50	1	389.50	41.02	0.0004	
B	53.64	1	53.64	5.65	0.0491	
C	70.17	1	70.17	7.39	0.0298	
A^2	1.64	1	1.64	0.17	0.6905	
B^2	13.40	1	13.40	1.41	0.2736	
C^2	1.47	1	1.47	0.16	0.7054	
AB	15.90	1	15.90	1.67	0.2367	
AC	9.59	1	9.59	1.01	0.3483	
BC	4.53	1	4.53	0.48	0.5120	
Residual	66.47	7	9.50			
Lack of Fit	44.60	5	8.92	0.82	0.6313	not significant
Pure Error	21.87	2	10.94			
Cor Total	652.06	16				

From the table 4.3 for RWGPE, the model F-value of 149.94 implies the model is significant. There was only a 0.01% chance that a "model F-value" this large could be occur due to noise. Value of "Prob > F" less than 0.0500 indicate the model terms are significant. In this case, A, B, C, are significant model terms and A^2, B^2, C^2, AB, AC and BC. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. The "lack of Fit F-value" of 4.97 implies there is a 5.16% chance that a "lack of Fit F-value" this large could occur due to noise. Lack of Fit is not significant is good model.

Table 4.4 Analysis of Variance (ANOVA) for the regression model equation and coefficients of WWGPE

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	Model significance
Model	834.42	9	92.71	6.11	0.0131	significant
A	545.09	1	545.09	35.93	0.0005	
B	88.45	1	88.45	5.83	0.0465	
C	120.20	1	120.20	7.92	0.0260	
A^2	9.32	1	9.32	0.61	0.4587	
B^2	5.48	1	5.48	0.36	0.5667	
C^2	0.89	1	0.89	0.059	0.8158	
AB	4.77	1	4.77	0.31	0.5923	
AC	15.40	1	15.40	1.02	0.3472	
BC	17.05	1	17.05	1.12	0.3243	
Residual	106.20	7	15.17			
Lack of Fit	59.17	5	11.83	0.50	0.7683	not significant
Pure Error	47.02	2	23.51			
Cor Total	940.62	16				

The model F-value of 6.11 implies the model is significant. There is only a 1.31% chance that a "model F-value" this large could occur due to noise. The Probability Values of "Prob > F" values less than 0.0500 indicate model terms are highly significant. In this case A, B, and C, was significant model terms. However, the interactions AB, AC and BC and A^2, B^2 and C^2 were insignificant. Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 0.50 implies the Lack of Fit is not significant relative to the pure error. There is a 76.83% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good we want the model to fit.

4.2 Effect of Extraction Process Variables

Based on the analysis of variance, the antioxidants extract yield was significantly affected by various interactions between the process variables. On the other hand, significant individual process variables that affect the extract yield are solvent ratio, time and temperature. These factors have been shown to influence the yield of extract. This result demonstrated the advantage of using design of experiments in capturing the interaction between variables that affects the extraction of antioxidants.

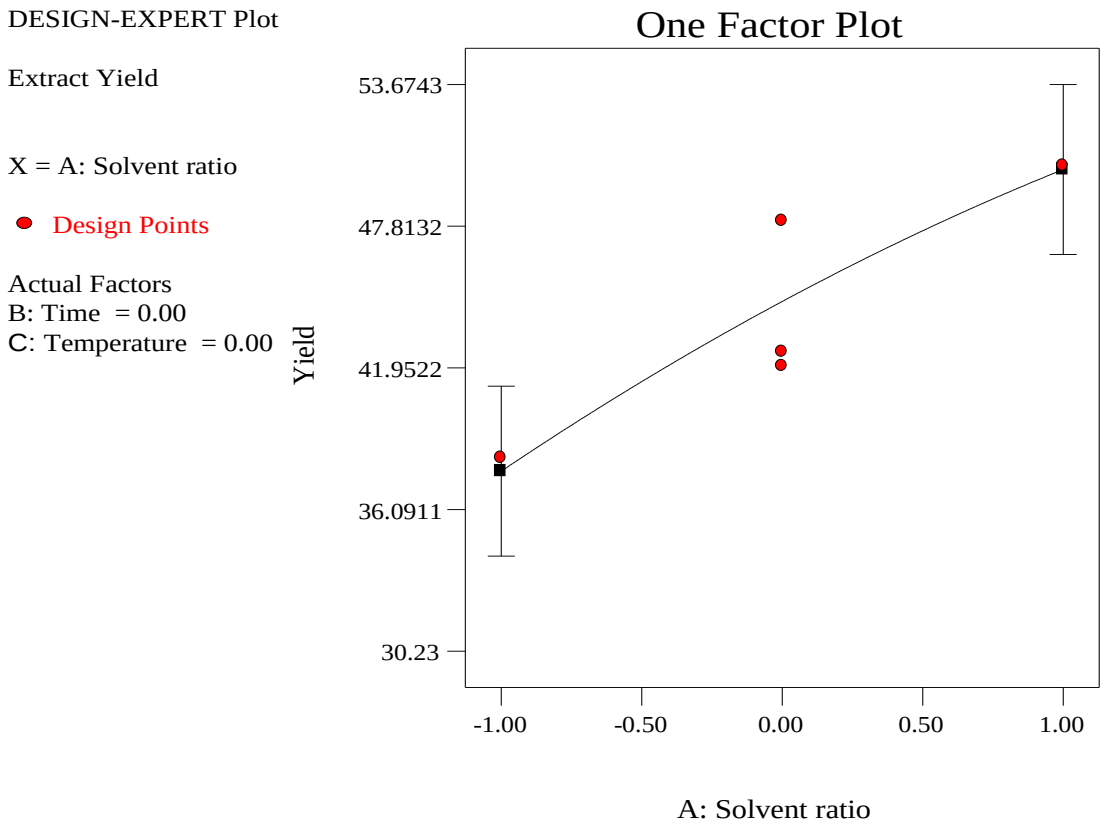
4.2.1 Effect of Individual Process Variables

4.2.1.1 Effect of Solvent Concentration on Yield

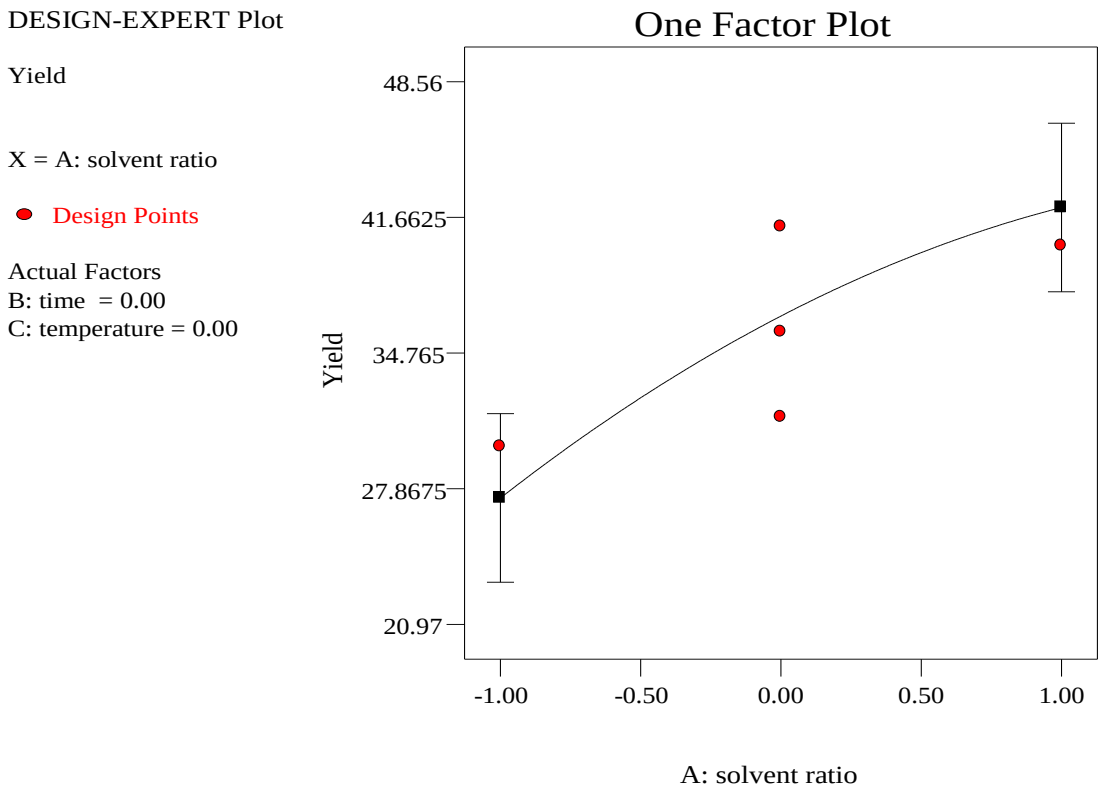
In fact, ethanol, a polar solvent, effectively extracts flavonoids and their glycosides, catecols and tannins from raw plant materials (Bazykina *et al*., 2002), but solubility of these compounds can be enhanced using a mixed solvent over a limited compositional range (Cacace and Mazza, 2003).

As observed in figure 4.9 A and B below for RWGPE and WWGPE, respectively decreasing of water content of ethanol was influent in improving extraction yield for both RWGP and WWGP, in total extract yield. This study confirmed that extract yield was improved decreasing the water percentage of ethanol from 40% to 10%, and, then, it did not make as such big change for water content between 40% and 25%, while total extract yield kept on decreasing with water content. Similar trends were reported by other authors, it was reported Yilmaz and Toledo (2006) that yield of extract of ethanol extracts from grape seed powder increased decreasing water in the mixture from 30% to 10%.In another study Cacace and Mazza, (2003) obtained that extraction of anthocyanins from black currants using aqueous ethanol increased with ethanol concentration up to a maximum at about 90%. In the same work it was suggested a different optimum ethanol content for the extraction of each group of phenols. According to figure 4.9 the total extract yield kept on decreasing with increasing water content, concentration of phenolic constituents in the extracts increased for water content from 40% to 10%, so a value of 10% was retained as the optimal one for both RWGPE and WWGPE. As shown in figure 4.9 (a and b) below for RWGPE and WWGPE, respectively the extract yield is significantly affected by solvent ratio or solvent concentration, it can be seen that with rising of the ethanol ratio and decreasing water ratio the

yield was generally increases. The reason is that as the ethanol ratio increases would increase the solubility of the extracted antioxidants, giving a higher rate of extraction. According to the principle of "like dissolve like", solvents would only extract those compounds which have similar polarity with the solvents (Spigno and De Faveri, 2007; Zhang and Wang, 2005). Increasing of ethanol concentration up to 90% was associated with increasing of antioxidant extract yield form 30.23% at lowest level coded (-1) ethanol ratio to water up to 52.35% at higher level coded (+1) of ethanol ratio for RWGP and also follow them same trend in case of WWGP from 20.97% to 48.56%. Based on these experimental results, it was believed that highly active phenolic compounds presented in Wine Grape Pomace were moderately polar. However, further increased the ethanol concentration from 60% from lowest level coded (-1) to 90% higher level coded (+1) in WWGP increases the extraction yield than RWGP. It was believed that this observation was due to extraction of different molecular weight of phenolic compounds in WWGP than RWGP.



(a) Effect of ethanol to water ratio on yield at fixed time and temperature for RWGP



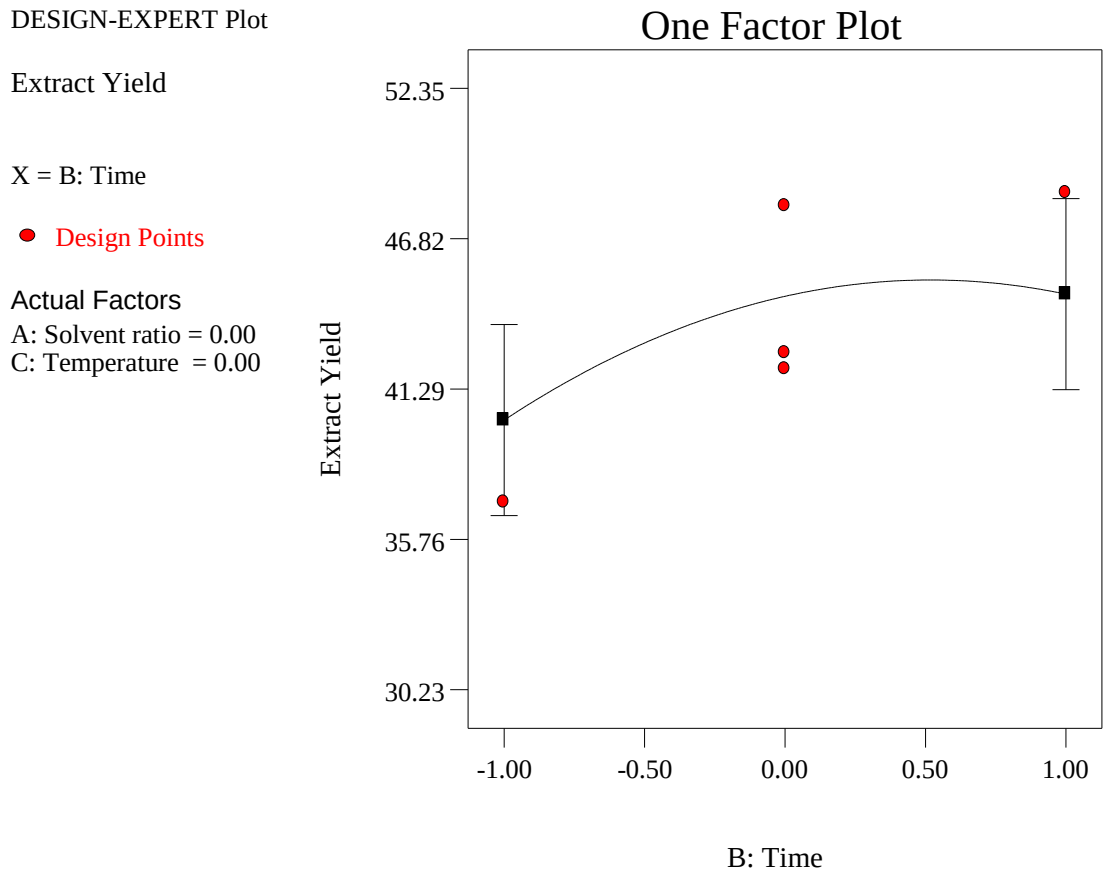
(b) Effect of ethanol to water ratio on yield at fixed time and temperature for WWGP

Figure 4.9 (a) and (b) effect of ethanol to water ratio on yield at fixed time and temperature for RWWGP and WWGP extracts ,respectively

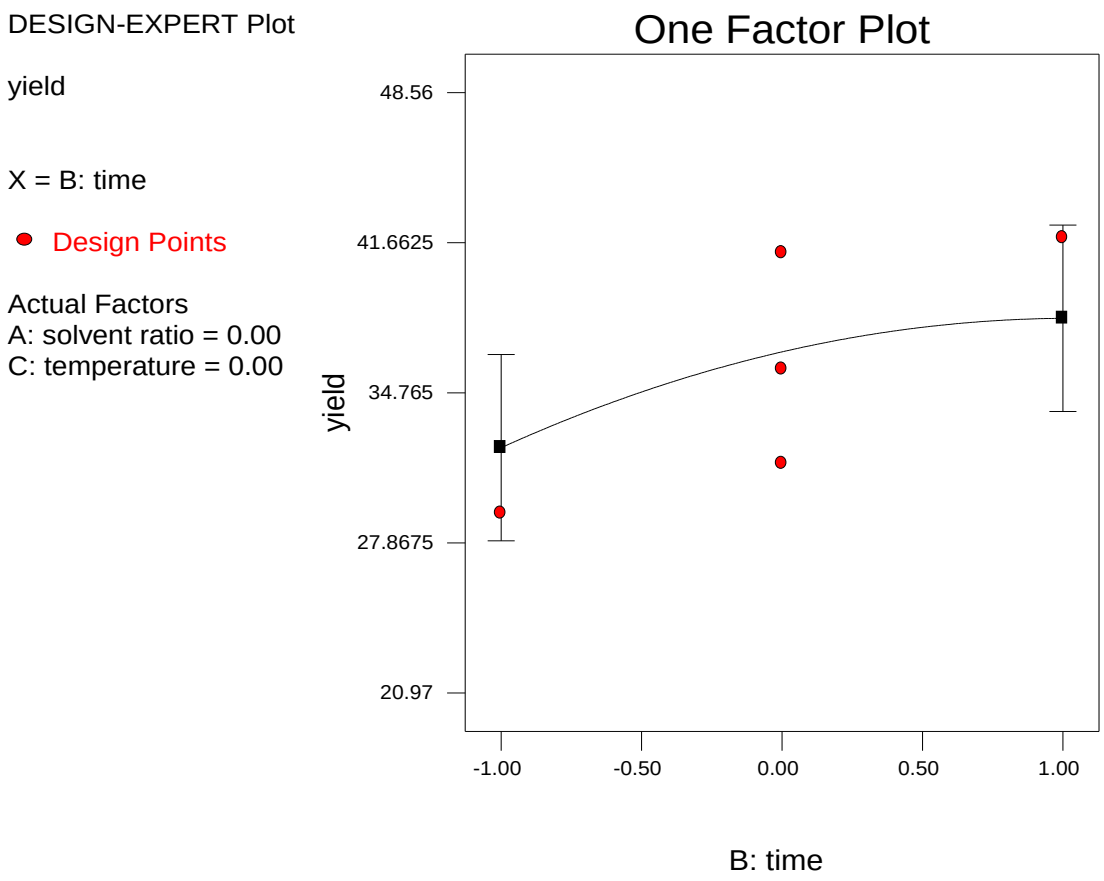
4.2.1.2 Influence of Extraction Time

Extraction time is crucial in solvent extraction of phenolic compounds as appropriate extraction time can result in time and cost saving. From figure 4.10 (a and b) below, as expected, a longer extraction time generally led to a higher percentage yield of extract for RWGP and WWGP extracts ,respectively. This could have been due to the longer amount of time the solute and solvent were in contact with each other. Longer contact time favored the system to have more mass transfer. However, excessive extraction time would be unnecessary as the solvent and sample would be in final equilibrium after certain duration. This is based on Fick's second law of diffusion by then; the rate of extraction of compounds would decelerate (Teixeira *et al*., 2014).The effects of extraction time on the yield of crude extract are showed in figure 4.10(a and b) for RWGP and WWGP, as shown in the figure 4.10, the highest extraction yield content

is obtained at extraction time of 120 min with a value of 52.35% for RWGP and 48.56 % for WWGP in both cases as extraction time increases yield increases as we move from lowest coded level (-1) or 60 minutes to highest coded level(+1) or 120 minutes for both RWGP and WWGP which shows the same trend as illustrated in figure 4.10 (a and b) this was in agreement with some literature results. It was found out that in water-extracts made of grape the yield of polyphenols gently increased with the time, while in the case of alcohol-extracts it strongly increased with the longer time of extraction (Lapornik *et al*., 2005). Other authors Pekić *et al*. (1998) reported that the kinetics curves of proanthocyanidins yield were of parabolic shape with the initial part being linear (up to 8h), whereas their second parts showed a slower increase and an asymptotic ending. In this study, the extraction time has a significant effect on the extraction yield of antioxidants and extraction yield were increased significantly with the increasing in the extraction time at fixed levels of both temperature and concentration as illustrated in the fig. 4.10



a) Effect of time on yield at fixed solvent ratio and temperature for RWGP



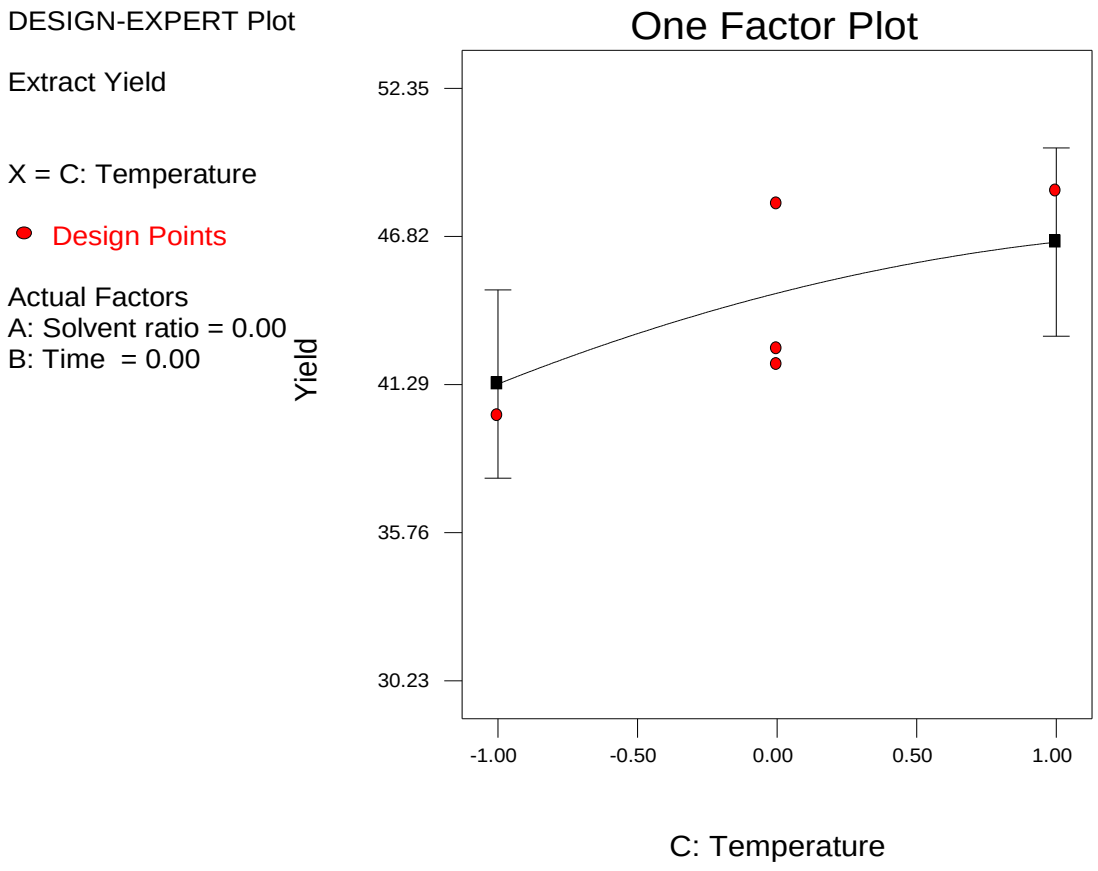
(b) Effect of time on yield at fixed solvent ratio and temperature for WWGP

Figure 4.10 (a) (b) effect of time at fixed temperature and solvent ratio for RWGP and WWGP respectively

4.2.1.3 Influence of Extraction Temperature

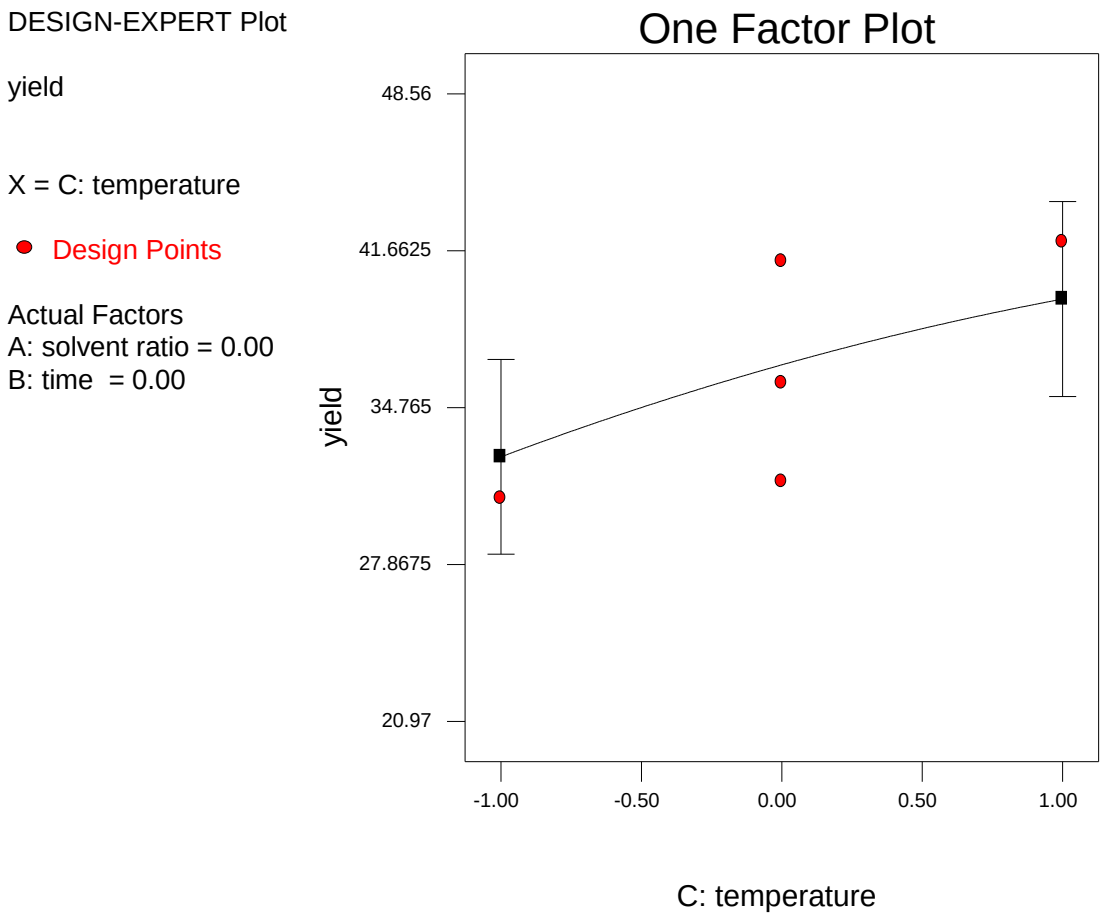
It can be observed in figure 4.11(a and b) that higher temperatures gave higher yields and, therefore, the highest extraction yield was obtained at 60 °C, for both RWGP and WWGP this was due to temperature strongly influent on extracts yield for both RWGP and WWGP, probably because temperature increase favored extraction by increasing solubility and diffusion coefficient of any compounds, not only of antioxidants. The extraction yield increases with increasing the extraction temperature (30°C - 60°C) (figure 4.11) ,according to Wells (2003) , an increase in temperature increases the efficiency of the extraction since heat render the cell permeable, increase solubility and diffusion coefficients of the compounds to be extracted and decreases the viscosity of the solvent, thus facilitating its passage through the solid substrate mass, these

authors found in their study on pomegranate's peel, that the use of temperatures higher than 60°C decreases the total polyphenols yield which is probably due to their degradation. Kim *et al.* (2006), in their work on rice explained this by the fact that the heat could solubilize the phenolic compounds but without breaking the covalent bonds of these compounds bound to the walls of the rice grains. The influence of temperature was investigated from Figure 4.11 it can be observed that the yield increases with the temperature from 30.23 % at 30 °C to 52.35 % at 60 °C for RWGP and similarly for WWGP it increases from 20.97% at 30°c to 48.56% at 60°c.This result is in agreement with the research work of Cruz *et al*. (2004) who, observed that increase in temperature enhances solvent extraction of the antioxidant compounds, thereby improving both diffusion coefficients and the solubility of a substance. In addition, this result showed that extraction temperature is an important factor to be considered in improving the efficiency of the extraction.



a)

a) Effect of temperature on yield at fixed solvent ratio and time for RWGP



b) Effect of temperature on yield at fixed solvent ratio and time for WWGP

Figure 4.11 (a) and (b) effect of temperature on yield at fixed solvent ratio and time for RWGP and WWGP, respectively

4.2.2 Effect of Interactive Parameters between Process Variables

The discussion of the single factors tells that even if each of the three factors has significant effect on the response of extract yield, but it was also observed the dependence of one another or their interaction. For graphical interpretation of the interactions between regressor variables, use of surface plots of the regression equation is highly recommended. Interaction implies that effect produced by changing one factor level (for example, time) depends on the level of the other factor. In this research, in order to investigate the interactive effect of independent variables on

the extraction condition from RWGP and WWGP, three dimensional (3D) plots were examined for the estimated response so three-dimensional response surface curves were plotted in order to understand the interactions between the variables and the optimum levels of each variables for maximum yield of extract from both RWGP and WWGP and also, contour curve presented the effect of two variables on the extract yield holding the third variable at constant level. The interaction between two variables namely, solvent ratio and time, solvent ratio and temperature, time and temperature are shown in Figure 4.12 to 4. 14 below, significance of interaction between the corresponding variable is indicated by saddle nature of the contour and 3D plots.

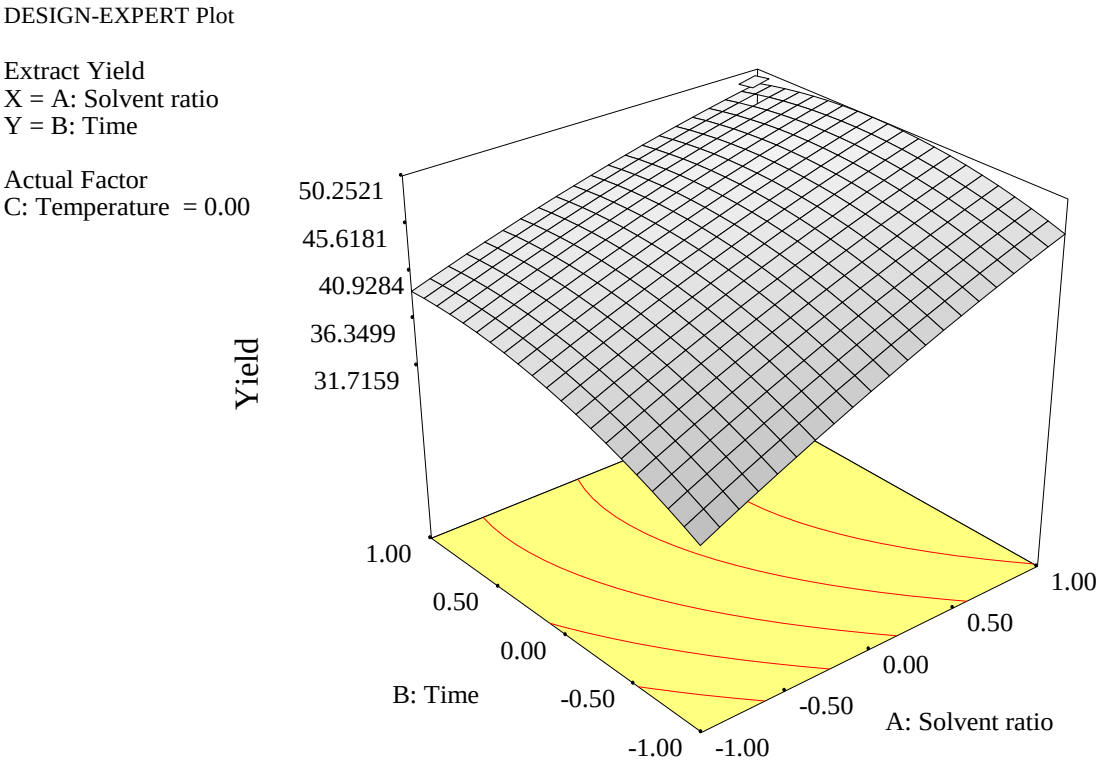
4.2.2.1 Effect of Solvent Ratio and Time on Yield

The effects of solvent ratio and time on the extraction of antioxidants extraction from RWGP and WWGP are show in the form of 3D and surface contour plots.

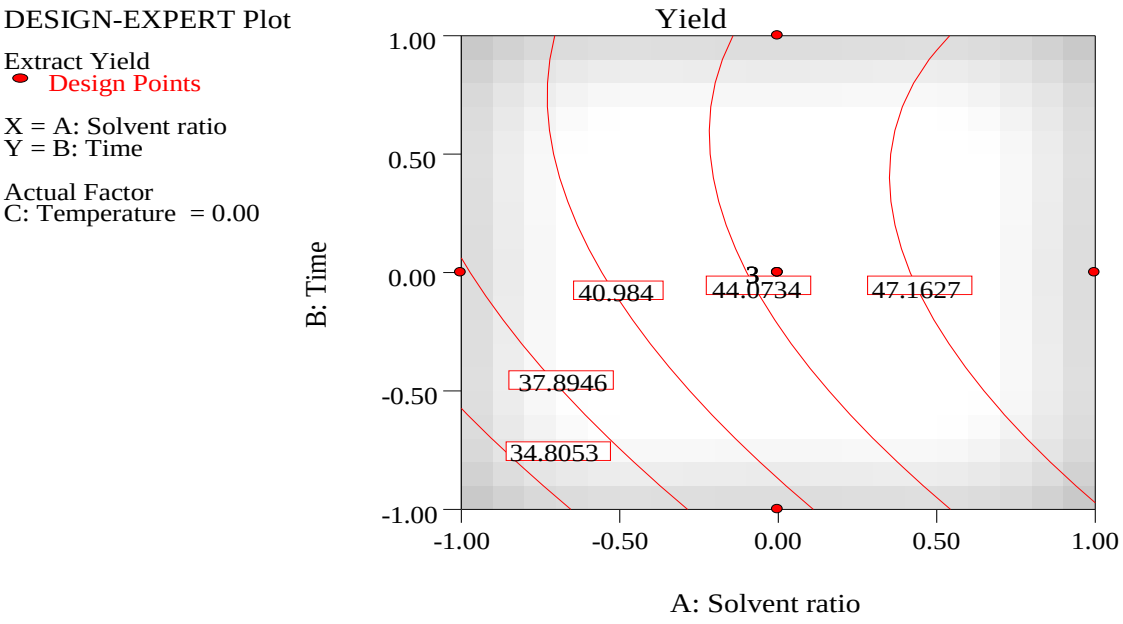
According to the results of table 4.2, the three dimensional response surface plots and surface contour were obtained, and shown in Figure 4.12. As depicted in Figure 4.12 below the dependence of antioxidants extraction from RWGP on both the solvent ratio and mix time, when temperature was at a fixed condition (45^{o}C) and similarly for WWGP extraction of antioxidants, at high level of solvent ratio the yield increase with increasing level of mix time from its low level to high level, hereafter the response become starts to increases slightly.

At high level of solvent ratio increasing level of reaction time from its low level to high level the response antioxidants extraction increases. Also at a given solvent ratio, increasing level of the mix time the response increases with the same trend in both RWGPE and WWGPE.

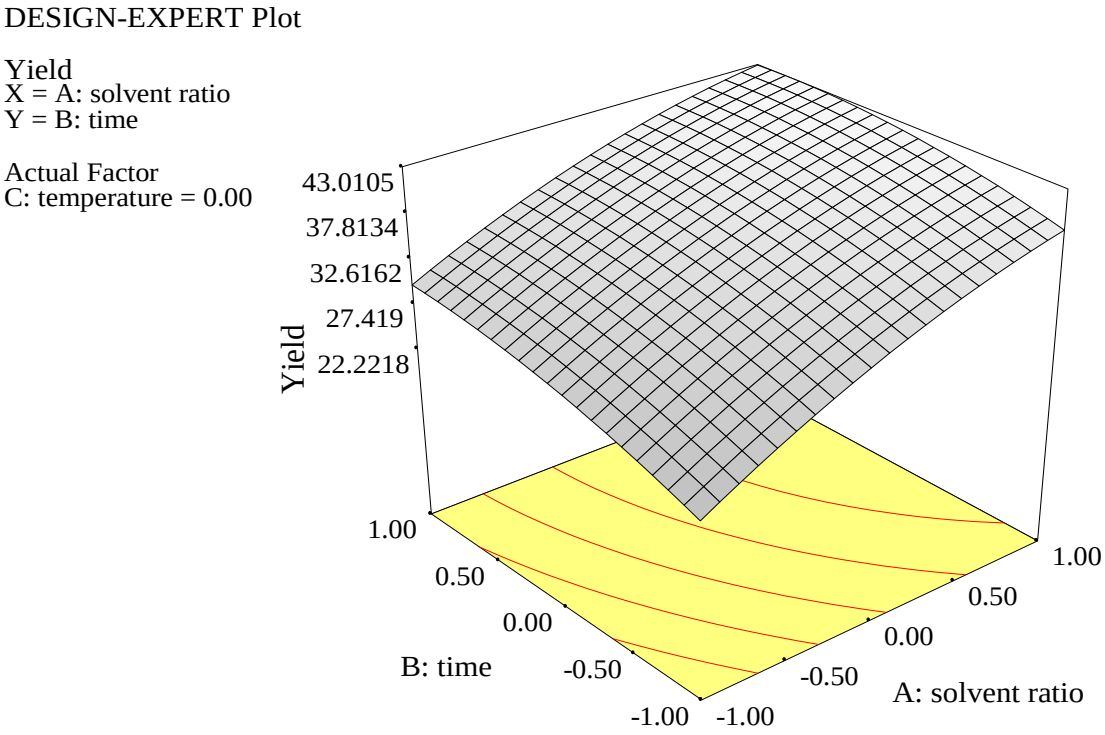
The contour response surface plots were able to illustrate the relationship between the independent variables and the response value. As shown in Figure 4.12(b and d), when the extracting temperature was fixed at center level, the response value (yield in percentage value) increased with increasing ethanol concentration over a certain range, but when the ethanol concentration was reduced to nearly 60%, the response value was decreased for both RWGPE and WWGPE. The response value increased with an increasing time. Generally as illustrated in figure 4.12 for both varieties the response yield increases as solvent ratio and time keep increasing together.



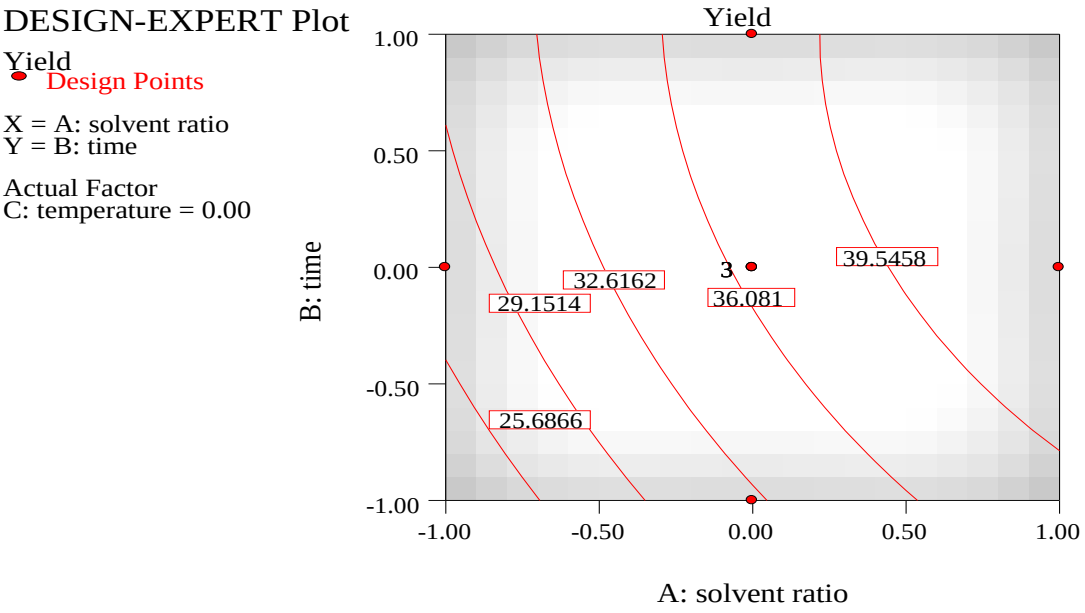
(a) 3D surface showing effect of solvent ratio and time on yield at fixed temperature for RWGP



(b) Contour plot showing effect of solvent ratio and time on yield at fixed temperature for RWGP



(c) 3D surface showing effect of solve ratio and time on yield at fixed temperature for WWGP



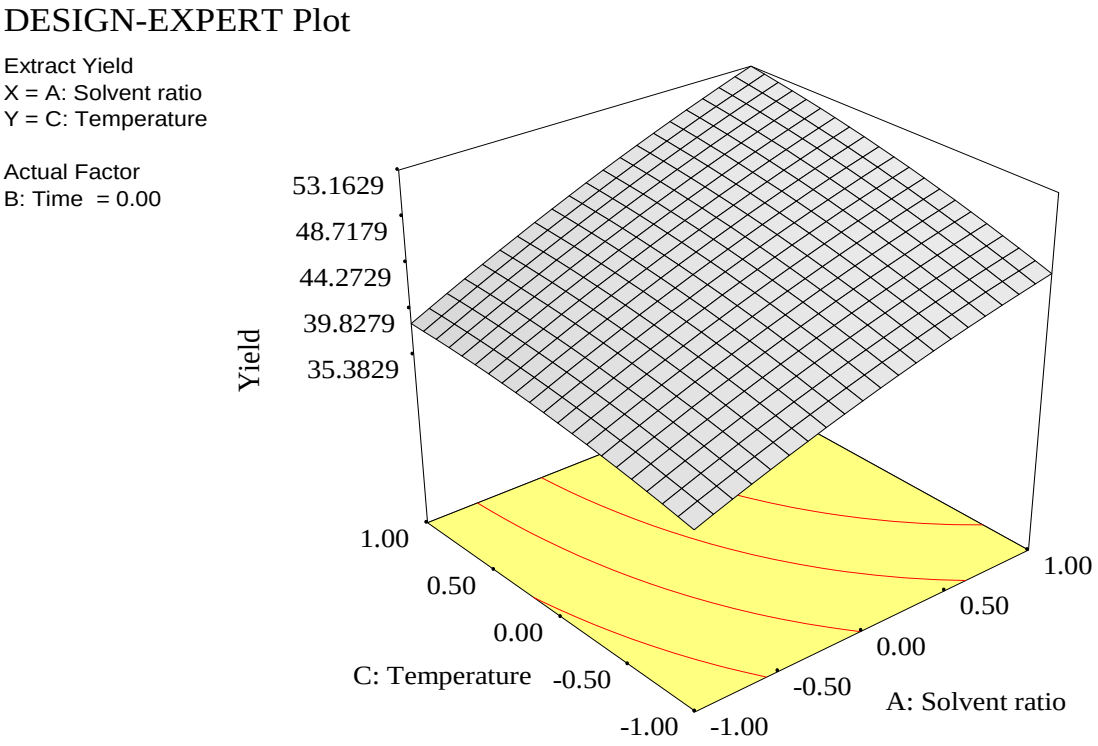
(d) Contour plot showing effect of solvent ratio and time on yield at fixed temperature for WWGP

Figure 4.12 (a), (b), (c) and (d) 3D surface and Contour plot showing effect of solvent ratio and time on yield at fixed temperature for RWGP and WWGP, respectively

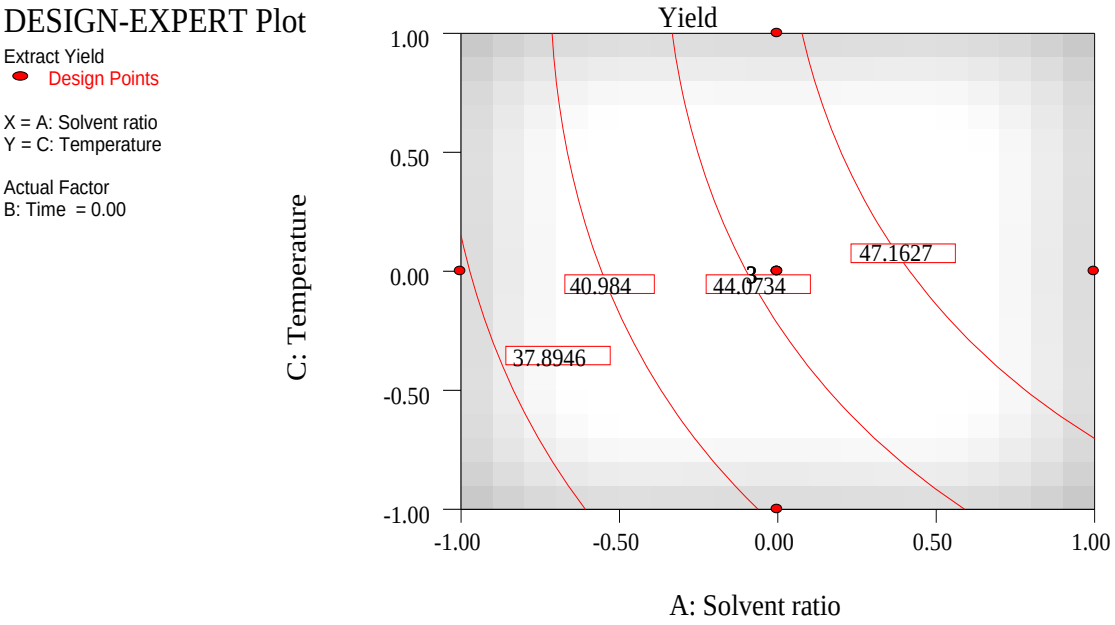
4.2.2.2 Effect of Solvent Ratio and Temperature on Yield

The interactive effects of solvent ratio and temperature on extraction yield of the two varieties of wine making by products were shown in the form of 3D plots and surface contour. The influence of solvent ratio and temperature on the response yield at fixed center level of extraction time was significant for both RWGP and WWGP extract as demonstrated in the figure 4.13 (a, b, c and d) below. The response yield on the contour plots predicted model indicated that at lower ethanol ratio and temperature, the amount of phytochemical compounds diffused towards the solvent was low due to low hydrolyzing rate of soluble phytochemical compounds at both low ethanol ratio and temperature. Hence, the extraction yield was low at low ethanol ratio and temperature for both RWGP and WWGP. The extraction yield was increased as increased in both ethanol ratio and temperature as shown in the figure 4.13 (a), (b), (c) and (d). Solubility of solute in solvent or mass transfer of solid to solvent is directly proportional with temperature. Increase in temperature could affect the yield by increasing the rate of solubility of phytochemicals (both water and ethanol soluble compounds) in ethanol-water mixture binary solvent. Hydrolysis of solid in solvent is also proportional with solvent concentration for RWGP and WWGP.

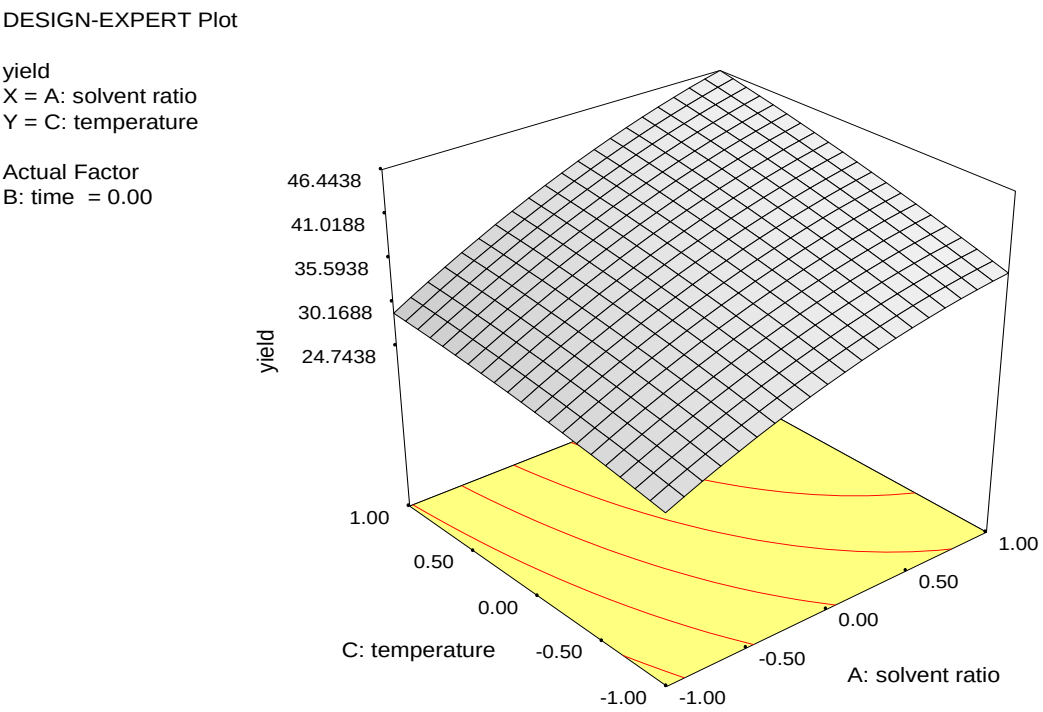
Extraction yield was affected by increased in ethanol concentration by facilitating hydrolysis of ethanol soluble phytochemical compounds (water soluble phytochemicals decreased). Extraction yield were limited to some interval of both solvent ratio and temperature due to degradation started at high temperature and concentration. At single contour surface line in Figure 4.13(b and d) for both RWGPE and WWGPE, the same response could be obtained by increasing the temperature and decreasing solvent ratio or vice versa.



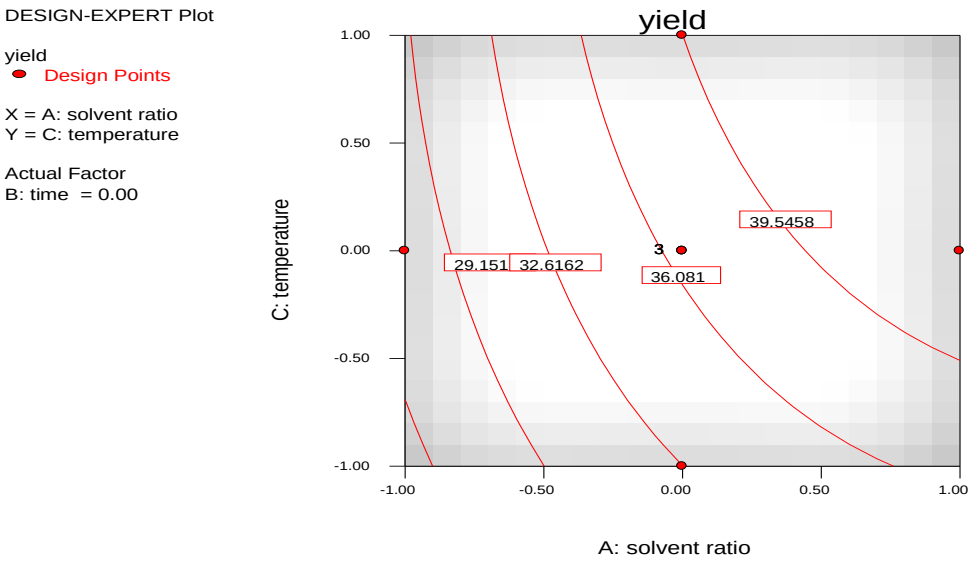
(a) 3D surface showing effect of solvent ratio and temperature on yield at fixed time for RWGP



(b) Contour plot showing effect of solvent ratio and temperature on yield at fixed time for RWGP



C) 3D surface showing effect of solvent ratio and temperature on yield at fixed time for WWGP

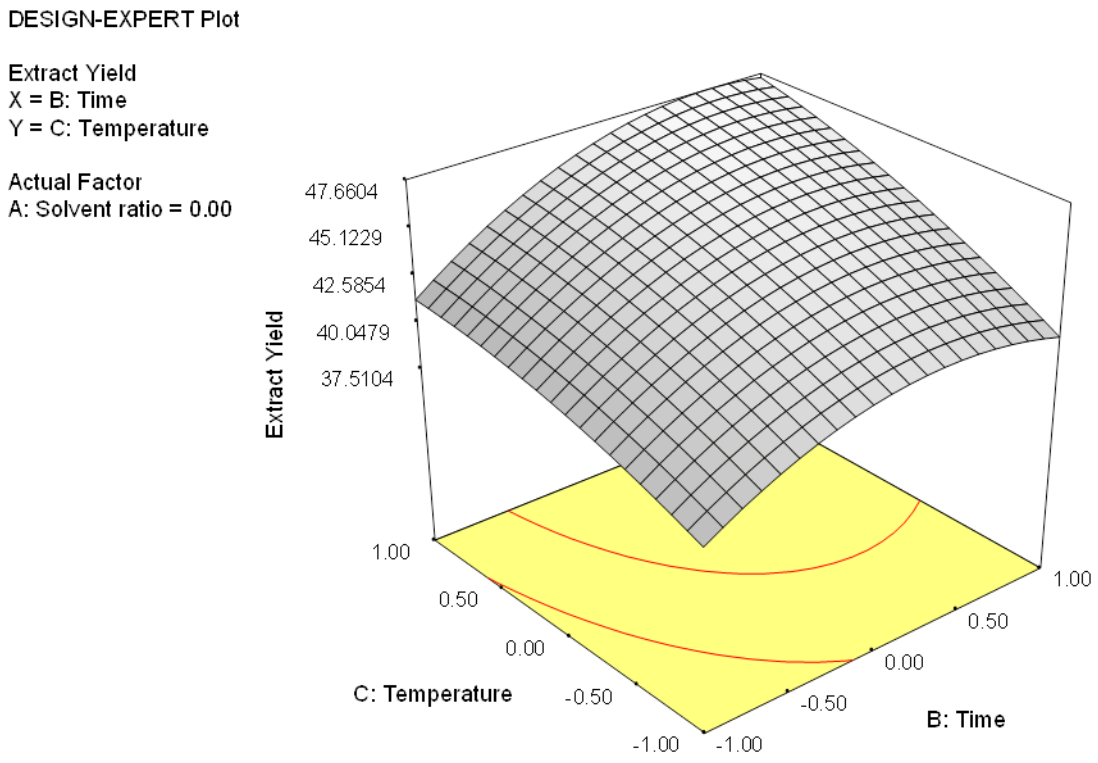


(d) Contour plot showing effect of solvent ratio and temperature on yield at fixed time for WWGP

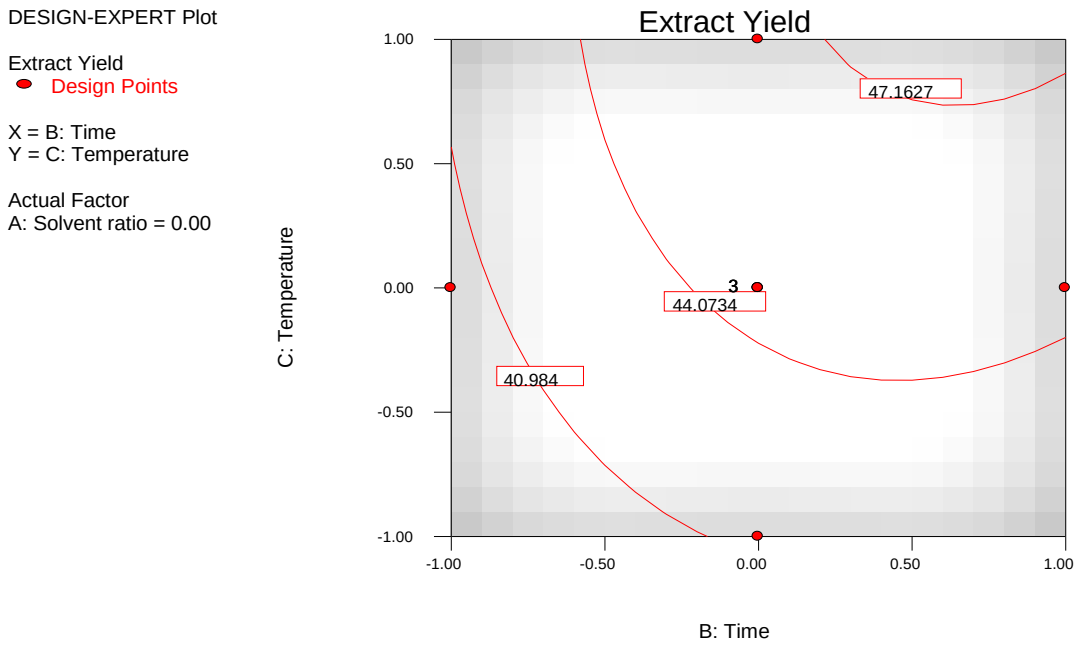
Figure 4.13 (a), (b), (c) and (d) 3D surface and Contour plot showing effect of solvent ratio and temperature on yield at fixed time for RWGP and WWGP, respectively

4.2.2.3 Effect of Time and Temperature on Yield

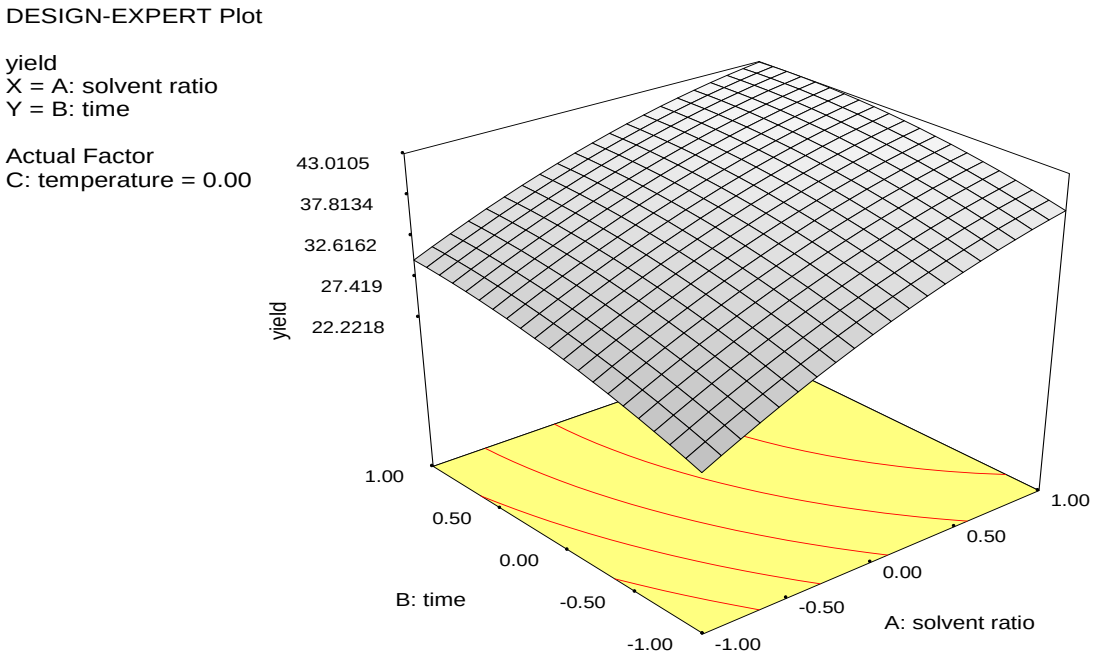
The effects of time and temperature on the extraction yield of antioxidants from RWGP and WWGP are show in the form of 3D plots and surface contour. As observed from Figure 4.14 (a) and (b) with rising of the extraction time and temperature the antioxidants yield were increased up to some optimal value but it declined when both temperature and time increased in case of WWGP. However, at moderate extraction time and high temperature the antioxidants yield are increased while at high extraction time the yield is slightly increases in case of RWGPE (Figure 4.14 (c) and (d)). Generally it was evident that the extraction temperature had a major role in the extraction of antioxidants from RWGP and WWGP when compared to the other two variables. This is due to both extraction temperature and times are two other key process variables in solvent extraction of antioxidants. From a pure mass transfer point of view, temperature increase favors extraction by increasing solubility and diffusion coefficient of any substance (Moure *et al*., 2001) and showing a positive effect of temperature on antioxidant activity of natural phenols (Pinelo *et al*., 2005).



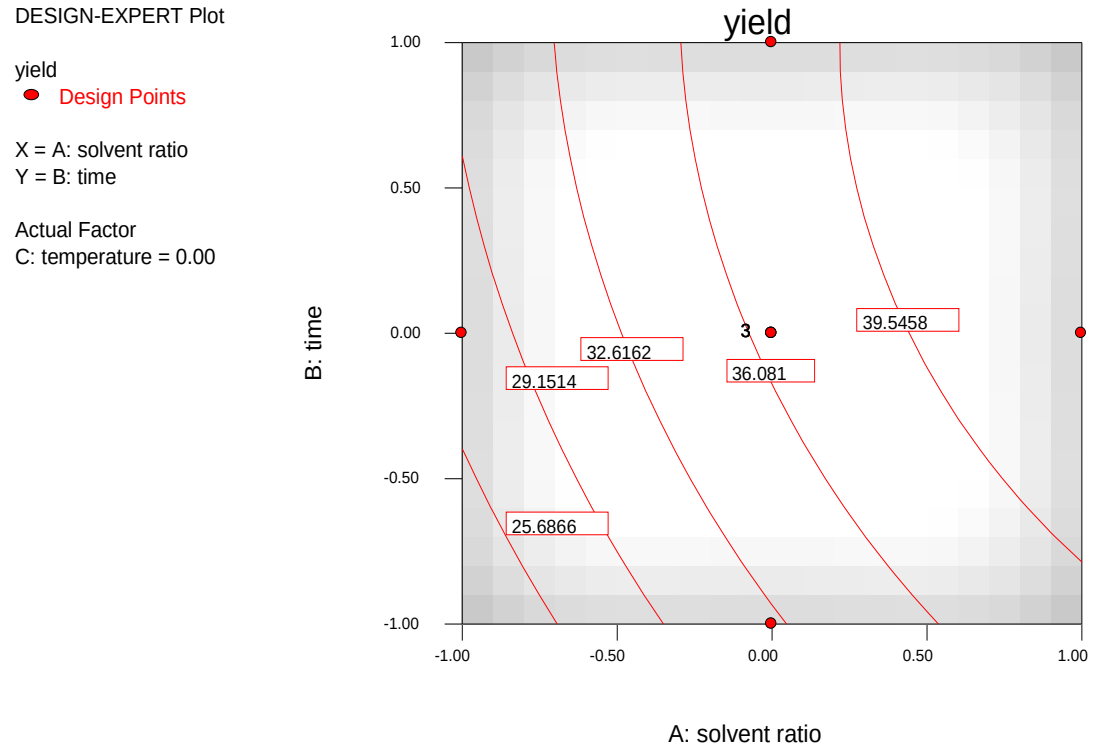
(a) 3D surface showing effect of time and temperature on yield at fixed solvent ratio for RWGP



(b) Contour plot showing effect of time and temperature on yield at fixed solvent ratio for RWGP



(c) 3D surface showing effect of time and temperature on yield at fixed solvent ratio for WWGP



d) Contour plot showing effect of time and temperature on yield at fixed solvent ratio for WWGP

Figure 4.14 (a), (b), (c) and (d) 3D surface and Contour plot showing effect of time and temperature on yield at fixed solvent ratio for RWGP and WWGP respectively

4.1.1 Optimization of Extraction Factors

The results above have shown that three extraction process variables and the interaction among the variables that affect the yield of antioxidants extract. Therefore, the next step is to optimize the process variables. From numerical optimization function in Design Expert 6.0.8, in order to obtain the highest yield using the model regression developed. The ethanol to water ratio, time and the temperature between them are highly and significantly affect the antioxidants extraction process. The optimization of process conditions affecting the percent (%) yield of extract were carried out for the maximum extraction of antioxidants from RWGP and WWGP using central composite design (CCD) method. Seventeen experiments with different combinations of solvent ratio, time and temperature were performed. The percent recovery of extract were determined; results was shown in Table 4.2 a detailed analysis on the interaction of solvent ratio, time and temperature on the % yield of extract antioxidants from RWGP and WWGP has been done.

The ‘Design-Expert 6.0.8(Stat-Ease, Inc., Minneapolis USA) software was used for regression and graphical analysis of the data obtained. The optimal values of the experimental conditions were obtained by solving the regression equation and also by analyzing the response surface and contour plots as shown above. The design expert gives three different optimization choices, numerical optimization (set goal for each response), graphical optimization set minimum and maximum limits for each response and then create an overlay highlighting an area of operability and point prediction optimization (enter desired operating conditions and discover predicted response values with confidence intervals). In numerical optimization choice, depending on constraints (criteria) selected, different alternative solutions of optimization were given by expert design. For this study, numerical optimization was selected to obtain better highest response yield for the RWGP and WWGP extracts.

4.1.1.1 Optimization of Extraction Factors for Red and White Wine Grape Pomaces

In the process of optimization criteria were set for the extraction process variables using an optimization function in Design Expert, in numerical optimization, there are criteria and constraints to be specified for process variables (solvent ratio/concentration, time and temperature,) and response yield. The goal of optimization should be set in range, minimum, maximum, target and equal to for factors and response variable. The limit (upper and lower), weight (upper and lower) and importance of all factors and response should be specified. The goals of solvent ratio, time, temperature and response yield all were set <in range> from lower and upper limits of their values. The upper and lower limits of the variables were specified and all variables were very significant and important due to their positive values from the developed regression model equation. The effect of a series of the factors on yield of extracts is shown in table 4.2 for both RWGPE and WWGPE. The optimum conditions were obtained by running the program of Design Expert software 6.0.8. Solvent ratio, Time and temperature of extraction are important parameter to be optimized even in order to minimize energy cost of the process. By considering minimum energy consumption and reduce cost of extraction the optimum conditions for independent variables and the predicted values of the responses also were presented as follows: for RWGPE solvent ratio (ethanol to water) 85.12:14.88 extraction time 99.03 minutes, extraction temperature 57.39 °C.

The estimated values for total extracts yield, 51.44 percent. Extractions were carried out at those conditions for RWGP. A verification experiment at the optimum condition, consisting of 3 runs, was performed and the practical yield of 47.74 percent of total extracts yield was obtained. And also for WWGPE solvent ratio (ethanol to water) 86.50:13.5, extraction time 95.10 minutes, extraction temperature 58.89 °C. The estimated values for total extracts yield, 45.50 percent. Similar to RWGPE extraction was carried out at those conditions for WWGP. A verification experiment at the optimum condition, consisting of 3 runs, was performed and the practical yield of 42.04 % total extracts yield was obtained. Sample from product of optimum condition for both RWGPE and WWGPE was selected for further characterization.

4.3 Characterization of Extracts

4.3.1 Color Intensity

The color intensity was determined by the content and structure of the anthocyanins present in a wine grape pomace extracts and is defined as the sum of the absorbance at 420, 520 and 620 nm calculated as per equation given in section three of this research equation 3.8 and 3.9 according to this as expected RWGPE has higher amount of anthocyanin than WWGPE the color intensity should be higher than WWGPE (table 4.5) below. The results for the color variables of the analyzed RWGPE and WWGPE were presented in table 4.5, from which it can be seen that the values of the color intensity was 4.789 for RWGPE for WWGPE 0.71 and it varied from one variety to another and the Hue or tone color was for RWGPE 0.450 and for WWGPE 0.254. The obtained results for the analyzed wines were in agreement with previously published data of Hadolin *et al*.(2004).

Table 4.5 color Intensity and Hue for RWGPE and WWGPE

Extracts	A_{420}	A_{520}	A_{620}	A_{280}	Color Intensity(I)	Hue
RWGPE	1.296	2.877	0.622	1.445	4.789	0.450
WWGPE	0.105	0.412	0.077	0.112	0.71	0.254

4.3.2 Total Phenol Content of Extract

To evaluate the extraction of phenolic compounds from the Wine Grape Pomace (WGP) residues, it was performed the quantification of Total Phenolic Content (TPC), using the Folin-Ciocalteau method table 4.6 and 4.7 for RWGPE and WWGPE, respectively. As shown in Table 4.7 and Table 4.8, both of the two tested pomace samples extract contained noticeable amount of phenolic compounds. The total phenolic content of the extract, calculated from the calibration curve (y=0.2655x+0.4777,R^2= 0.9533), was expressed as Gallic acid equivalents/g (GAE/g) in dry base. For RWGPE, the highest TPC obtained was 56.72 mgGAE/g extract, obtained at 12mg/ml concentration of sample. The results at 10, 8, 6 and 4 mg/ml concentration of sample, obtained are 53.10, 50.78, 31.34 and 15.70 mgGAE/g extract and total phenolic content of extract in RWGPE were increases with concentration of the extract. The change in concentration of the extract from 4mg/ml to 12 mg/ml leads to a slight increases in TPC as shown in Table 4.7. In case of WWGPE it shows the same trend as RWGPE that is as the concentration of the extract increases the TPC increases, the highest TPC obtained was 50.56 mgGAE/g extract (table 4.8) at maximum extract concentration that is 12mg/ml when we look for other extract concentration 4, 6, 8and 10mg/ml the TPC increases from 10.94, 24.12, 46.04 and 47.24 mgGAE/g extract for 4, 6, 8 and 10 mg/ml of extract sample concentration.

Table 4.6 Concentrations of Gallic acid standard solution and their corresponding absorbance

Concentration (mg/ml)	Absorbance(λmax=765nm) Mean ± SD
0	0
1	0.950 ±0.242
2	1.072 ±0.102
4	1.749 ±0.072
6	2.092 ±0.038
8	2.831 ±0.071
10	3.176 ±0.102
12	3.366 ±0.061

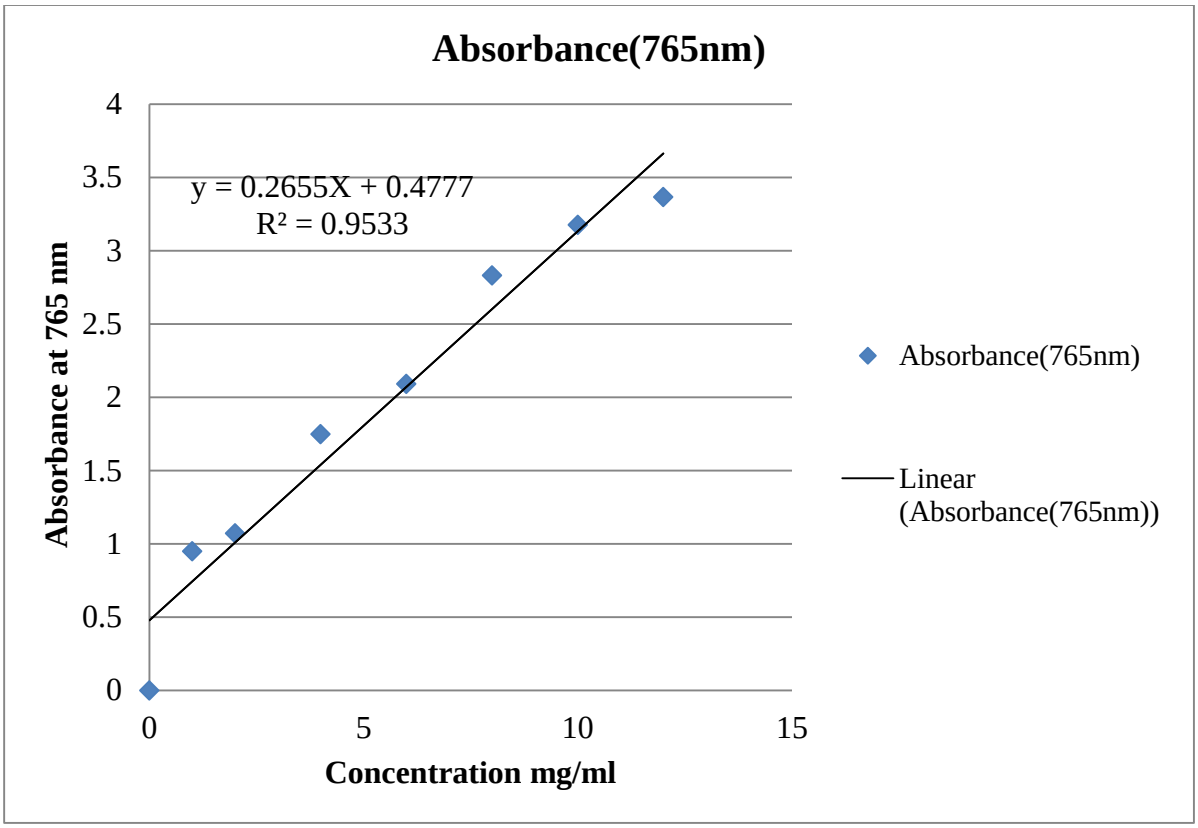


Figure 4.15 Gallic acid standard calibration curve

Table 4.7 Amount of total phenol content from a gram of dry Red wine grape pomace extract

Concentration of sample (mg/ml)	Absorbance (λmax=765nm) Mean ± SD	Gallic acid equivalent Concentration (mg/ml)	Total phenol content (mg GAE/g extract)
1	0.195±0.071	-	-
2	0.424±0.106	-	-
4	.0.685±0.071	0.785	15.70
6	0.893±0.040	1.567	31.34
8	1.151±0.102	2.539	50.78
10	1.182±0.102	2.655	53.10
12	1.230±0.118	2.836	56.72

Table 4.8 Amount of total phenol content from a gram of dry White wine grape pomace extract

Concentration of sample (mg/ml)	Absorbance (λmax=765nm) Mean ± SD	Gallic acid equivalent Concentration (mg/ml)	Total phenol content (mg GAE/g extract)
1	0.123±0.014	-	-
2	0.389±0.089	-	-
4	0.623±0.101	0.547	10.94
6	0.798±0.045	1.206	24.12
8	1.089±0.101	2.302	46.04
10	1.105±0.125	2.362	47.24
12	1.149±0.053	2.528	50.56

The results of content of total polyphenols of grape waste extracts, obtained from grape pomace after winemaking, and as shown in table 4.7 and 4.8. The obtained results showed significant Polyphenolic contents in both tested samples, ranged from 15.70 to 56.72mgGAE/g in RWGPE and 10.94 to 50.56mgGAE/g in WWGPE, in agreement with literature data (Atanackovic *et al*., 2012) and also similar to that found in *V. Vinifera var*. Bangalore blue pomace (Moure *et al*., 2001) . The extract from Red Wine Grape pomace Extracts (RWGPE) had the highest TPC, followed by White Wine Grape Pomace Extracts (WWGPE). Both pomace extract examined in the present study had higher TPC (56.72 mg GAE/g) and TPC (50.56 mg GAE/g), for RWGPE and WWGPE respectively compared to those reported by Kim *et al*.(2006), TPC of 30.4 mg GAE/g for RWGPE and 24.23mgGAE/g for WWGPE. The differences could arise from variations in genetic backgrounds, environmental factors, agronomic practices, or vinification processes (Boussetta and Vorobiev,2014).

The RWGPE contained the highest TPC (56.72 mg GAE/g) followed by the WWGPE (50.56 mg GAE/g) this may be due to, the phenolic composition in grape red and white varies widely and is usually determined by several factors, such as, the variety of grape and conditions under which they was grown (soil, geographical location, light exposure, temperature, sun exposure of the clusters, location of growth, ripening time) and other factors (Kim *et al*., 2006) and also, researchers found that the methods for wine making products from grape pomace vary depending on type of the grape pomace if the pulp is from red or white grapes because winemaking

processes differ for each varietal and they produce pomace with different levels of sugar, nitrogen, phenolics and other compounds. The other reason total phenolic of red grape pomace is higher than that of white grapes due to the loss of the ability to produce anthocyanins in the skins of white grapes. The results of this research showed that the phenolic content of different grapes depends mainly on the grape variety from red to white wine grape pomace.

The plants produce phenols as a response to the negative impacts from the environment as well as UV radiation, various pathogens, fungi etc., also the amount and types of phenol compounds present in a particular grape waste extracts can vary and is greatly influenced by the extraction in the winemaking process (Spigno and De Faveri, 2007) also, the amount and types of phenol compounds present in a particular grape pomace extracts can vary and is greatly influenced by the extraction in the winemaking process (Spigno and De Faveri, 2007). It has been noted that the TPC of the RWGPE was lower than that in commercial grape seed extract (80.70 g GAE/ g seed) reported by Alonso *et al.* (1991) and higher than in seeds of red grape varieties cultivated in Turkey (7.90-15.46g GAE/100 g seed) (Boussetta and Vorobiev, 2014). On the other hand, the data for the WWGPE from the present study were higher than those reported previously for Cabernet Sauvignon, Merlot and Shiraz red grape skins (Alonso *et al*.,1991). However, in the present study, sugars, proteins and pigments were not removed prior to testing, which may have added to the high values seen when detecting the total phenolic content. In addition, the discrepancies may be due to differences in cultivar, cultivation site, climate, viticultural technique and harvesting time.

Furthermore, Yang and Ahmedna, (2013) reported fresh Cabernet Franc grape extracts had TPC of 42 mgGAE/g, and fresh Vidal Blanc grape extracts had TPC of 23mgGAE/g significant differences in TPC between pomaces and fresh grapes are attributed to the localization of phenolic compounds mainly in the skin and seeds of grapes, depending on the grape cultivar, vintage, geographical origin, winemaking practices, and extraction methodology. In any case, all total phenolic values described in Table 4.7 and 4.8 for both RWGPE and WWGPE, respectively were in broad agreement with the aforementioned bibliographic range. In particular, a similar total phenolic content was presented 54.90 mgGAE/g by Lapornik *et al*. (2005) for Premsal Blanc pomaces. In the case of seed byproducts, Mendes *et al*. (2013) reported total phenolic values, for both Italian Riesling and Rhine Riesling white varieties, slightly lower than those

described for white pomaces considered in the present study. Results of this research were in agreement to those of Bazykina *et al.* (2002) who mentioned the presence of moderate phenol amounts (50.11-105.7 mgGAE/g DW) of four international varieties from Turkish Vitisvinifera pomace. However, Lu *et al*.(1999) noted that total phenolic content in pomace of *Vitisvinifera L*. species can reach 667.98 mgGAE/g dry weights. These variations in total phenol content could be due to the various factors. One such factor may be the genetic potential of individual species for polyphenol biosynthesis (Alonso *et al*., 1991). Apart from the genetic (varietal) background, maturation stage may also be critical in this respect (Atanackovic *et al*.,2012).

4.3.3 Total Flavonoid Content of Extract

The total flavonoid content (TFC) for RWGPE and WWGPE were measured with the aluminum chloride colorimetric assay using cathechin as standard. Aluminum chloride forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxide group of flavones and flavonols. In addition it also forms liable complexes with orthodihydroxide groups in A/B rings of flavonoids. The calibration curve for eleven sequentially and independently prepared stock standard solutions of cathechin that depicts the concentration of cathechin against the absorbance, presented in Figure 4.16. The absorbance value increased proportionally upon increasing the concentration of cathechin from 0.0 to 0.5 mg/ml. A slight deviation from the linearity seemingly occurred at the higher concentration region of cathechin calibration plot. Nevertheless, for estimation purposes, the calibration plot was employed to ascertain the total flavonoid content of both RWGPE and WWGPE. Total flavonoid content in the RWGPE was found to be 49.659mgCE/g, that is to say 1 g of the extract contains 49.659 mg of cathechin equivalent and for WWGPE it was found 18.567mgCE/g.

The cathechin solution of concentration (0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45 and 0.5 mg/ml) conformed to Beer's Law at 510 nm with a regression coefficient (R^2) = 0.9999. The plot has a slope (m) = 4.2955 and intercept = 0.0109.The equation is y = 4.2955x + 0.0109 (Figure 4.16). According to table 4.10, the content of total flavonoids (in terms of dry base sample) of the Red wine grape pomace extract (RWGPE) (49.659mg CE/ g) was significantly higher than the White wine grape pomace extract(WWGPE) (18.567mg CE/ g) and these results were not correlated with total phenolics in this research. As discussed above the TFC of RWGPE sample is 49.658 mg CE/g at sample concentration of 1mg/ml and WWGPE is 18.567 mg CE/g

at sample concentration of 0.5mg/ml. These results suggested that RWGPE was best than WWGPE selected for extracting flavonoids from wine grape pomace (WGP).

Table 4.9 Concentrations and Absorbance for catechin standard calibration curve

Catechin Concentration (mg/ml)	Absorbance (λmax=510nm) Mean ± SD
0	0
0.05	0.234±0.123
0.10	0.448±0.162
0.15	0.658±0.019
0.20	0.869±0.098
0.25	1.083±0.117
0.30	1.294±0.090
0.35	1.504±0.073
0.40	1.734±0.224
0.45	1.954±0.093
0.5	2.154±0.079

The values of the total flavonoid content in the RWGPE and WWGPE were lower than those reported for the varieties Merlot (122.70 mg CE/g db) and Cabernet (125.00 mg CE/g db) (Boussetta and Vorobiev, 2014) and for other WWGPE (102.58 mg CE/g db) (Makris *et al*., 2007). The difference is presumably due to the extraction method, which might have caused partial degradation of the flavonoids, and may be due to the different variety and source of grapes. Likewise, TFC values for the RWGPE were higher than those reported previously for red grape peel (35.87 mg CE/g db) (Makris *et al.*, 2007) and five wild grapes and two hybrids native to Japan (0.3-3.4 mg QE/g) (Prozil *et al*., 2012). Ascribed, the difference mainly to the type of extraction method employed. However, quantitative differences might also be a factor of cultivar, cultivation site, climate and viticultural technique. As appreciable from table 4.10, extracts demonstrated that RWGPE has higher flavonoid than WWGPE which shows RWGPE may has high antioxidant capacity, suggesting that total flavonoids from different types of wine by products were significantly different depending again on extracting methods and sources of

grape pomace. The grape processes which provided grape pomace byproducts have affected total phenolic and total flavonoid contents which were consistent with the previous study by Bravo, (1998).

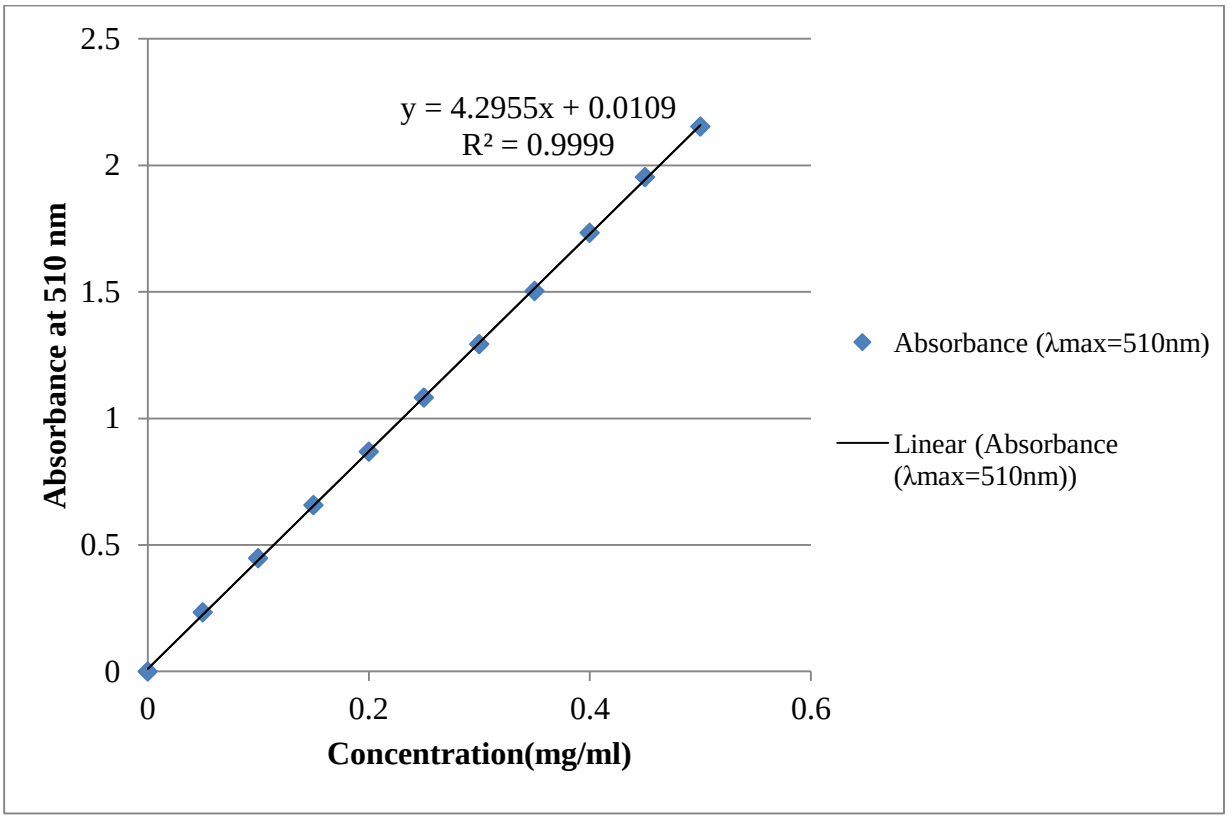


Figure 4.16 Catechin Standard linear calibration curve

Table 4.10 Absorbance and Total flavonoid content of red wine grape pomace and white wine grape pomace extracts

Grape Pomace	Concentration (mg/ml)	Absorbance (λmax=510nm) Mean± SD	Catechin Equivalent Concentration (mg/ml)	Total flavonoid content (mg CE/g extract)
RWGPE	1	0.856±0.003	0.1965	49.659
WWGPE	0.5	0.0588±0.025	0.0109	18.567

4.3.4 Total Anthocyanin in Extracts

The total anthocyanin concentration and the total amount of anthocyanin present in the sample were determined by using the pH differential method in which Absorbance observed determined according to Equation 3.13. After obtaining the absorbance values the anthocyanin pigment concentration in the original sample was calculated by using the formula in Equation 3.14. Extinction coefficients of some anthocyanins were reported in literature, but if the identify was unknown, it will indicate the use of cyanidin-3-glucoside extinction coefficient, since it was the most abundant anthocyanin in nature. DF is dilution factor, which was equal to 37.5 for all samples (DF was calculated as followed; 1gm of extract was dissolved in 10ml solvent, 0.8ml of this solution was taken and the volume was completed to 3ml with buffer pH=1, so the dilution factor = 3 / 0.8 = 3.73; (DF =3.73 x 10 = 37.5). The values obtained were listed in Table 4.11. The absorbance measurements at two different pH values were possible because of the structural transformation of anthocyanin as a function of pH. The absorbance was measured at 530 nm at pH=1 because at these conditions, anthocyanins have the maximum absorption, and so do other compounds that might be present in the sample, also the absorbance was measured at pH=4.5 because at this pH anthocyanins solution were colorless and there was no absorption, so other compounds that had absorbed at pH=1 would still absorbed at pH=4.5 while the anthocyanins does not. By measuring the absorbance at 700 nm and subtract from λmax in equation 3.13 the absorbance was corrected for other compounds which might have absorbed at pH=1 and pH=4.5. By this equation it was able to calculate the amount of anthocyanin which was obtained from both RWGPE and WWGPE.As expected, the anthocyanins were high in the RWGPE (530 mg/L) (table 4.11) and low in the WWGPE (14.01mg/L), due to the fact that they are mainly stored in the vacuoles of the exocarp (peel) cells of grapes. The results indicate that the total anthocyanin content (TAC) is localized predominantly in the skins of grapes. These findings are consistent with data published previously (Yilmaz and Toledo , 2006), despite differences in extraction method.

Table 4.11 Absorbance and Total anthocyanin content of red wine grape pomace and white wine grape pomace extracts

Extracts	λmax (nm)	pH=1		pH=4.5		Absorbance correction	Anthocyanin concentration (mg/L)
		λmax	A_{700}	λmax	A_{700}		
RWGPE	540	1.250	0.025	0.3797	0.001	0.052	530
WWGPE	530	0.580	0.06	0.525	0.025	0.263	14.01

From Table 4.11 it is observed that RWGPE (530 mg/L) had the highest total anthocyanin than WWGPE (14.01 mg/L) which confirms results reported by Spigno *et al*.(2007), this large difference is due to Anthocyanins (in red pomace) and flavanols are the most abundant in wine pomace, leaving all others in a minority. According to the normal composition of *Vitisvinifera* red varieties, the predominant anthocyanin is malvidin-3-*O*-glucoside that is usually followed by peonidin, petunidin, or delphinidin-3- glucoside depending on the grape variety (Gonzalez-Montelongo *et al*., 2010),depending on the species the anthocyanin content ranges from 500 mg kg^{-1} up to 3 g kg^{-1}. Red and white varieties present remarkable differences in anthocyanin composition because light plays a crucial role in the phenolic metabolism (Makris *et al*., 2007). Furthermore, anthocyanins are considered as potential substitutes for synthetic colorants owing to their bright, attractive colour and water solubility, which make them attractive for incorporation into a variety of food systems (Alonso *et al*., 1991).Considering grape pomace as a source for extracting natural colorants like anthocyanins, the red grape pomace has a greater potential than white grape pomace varieties evaluated in this study.

4.3.5 Radical Scavenging Activity of Extract

Radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biological systems. Chemical assays are based on the ability to scavenge synthetic free radicals, using a variety of radical-generating systems and methods for detection of the oxidation end-point. DPPH radical scavenging methods are common spectrophotometric procedure for determining the antioxidant capacities of components. DPPH, a pragmatic compound with an odd electron, shows strong absorption band at 517 nm in methanol.

Free radical scavenging activity or inhibition activity of free radical DPPH percentage (IA %) of standard Ascorbic acid, RWGPE and WWGPE was calculated using the relationship written in the equation 3.8 of section three. As shown in Table 4.12, 4.13 and 4.14 below the extract were capable of neutralizing the DPPH free radicals via hydrogen donating activity by 42.27, 74.17, 89.33, 95.91, 98.10, 98.40 and 98.50% for RWGPE and for WWGPE 39.10, 61.12, 71.11, 79.56, 84.04, 86.14 and 88.23% at concentrations of 1, 2, 4, 6, 8, 10, and 12 mg/ml, respectively. Table 4.12, 4.13 and 4.22 shows IC_{50} value of ascorbic acid, RWGPE and WWGPE were determined from the regression line of concentration versus % of inhibition and IC_{50} value of ascorbic acid (2.67 mg/ml), RWGPE (1.24 mg/ml) and for WWGPE (1.49 mg/ml)(dry weight).

As shown in Figure 4.17, DPPH scavenging was increased in a concentration dependent manner. The absorbance decreases as the result of color change from purple to yellow due to the scavenging of free radical by antioxidants through donation of hydrogen to form the stable DPPH-H molecule (Moure *et al*., 2001). The antioxidant activity of two different extracts from the RWGPE and WWGPE is expressed in terms of percentage of inhibition (%) and IC_{50} values (mg/ml) and A_{AR} (table 4.13 and 4.14) and for standard ascorbic acid at table 4.12 for comparison. Parallel to examination of the antioxidant activity of both wine grape pomace extracts, the values for standard compound were obtained and compared to the values of the antioxidant activity. The examination of antioxidant activities of plant extracts from RWGPE and WWGPE showed different values. The obtained values varied from 42.27% to 98.50% for RWGPE and for WWGPE it varied form 39.10% to 88.23%. The largest capacity to neutralize DPPH radicals was found for RWGPE extract, which neutralized 98.50% of free radicals at the concentration of 12 mg/ml. A moderate activity was found for WWGPE, extracts which was 88.23%. In comparison to IC_{50} values of Ascorbic acid, RWGPE and WWGPE extract from Awash Winery manifested the strongest capacity for neutralization of DPPH radicals but when compare all RWGPE possess the highest value. Numerous investigations of qualitative composition of plant extracts revealed the presence of high concentrations of phenols in the extracts obtained using polar solvents and the extracts that perform the highest antioxidant activity have the highest concentration of phenols (Chamorro *et al*.,2012) from table 4.7 and table 4.8 which were agreed with previous analysis of TPC for both RWGPE and WWGPE in this research. Numerous investigations of the antioxidant activity of plant extracts have

confirmed a high linear correlation between the values of phenol concentration and antioxidant activity (Kammerer *et al*., 2005). RWGPE extracts have high concentration of total phenols (table 4.7) and flavonoids (table 4.8), which is in correlation with intense antioxidant activity of these extracts.

Table 4.12 Average absorbance of ascorbic acid and corresponding concentration

Ascorbic acid concentration (mg/ml)	Absorbance(λmax=517nm) Mean± SD	% IA	IC_{50} (mg/ml)	A_{AR}
Control(0)	1.003	-	-	
1	0.773±0.041	22.92	2.67	0.37
2	0.588±0.065	41.29		
4	0.330±0.042	67.07		
6	0.092±0.070	91.05		
8	0.085±0.014	91.73		
10	0.063±0.340	93.68		
12	0.037±0.005	96.27		

Table 4.13 DPPH absorbance of sample concentration for Red Wine Grape Pomace extract

Sample Concentration (mg/ml)	Absorbance(λmax=517nm) Mean ± SD	% IA	IC_{50} (mg/ml)	A_{AR}
1	0.579±0.057	42.27	1.24	0.80
2	0.259±0.029	74.17		
4	0.107±0.001	89.33		
6	0.041±0.004	95.91		
8	0.019±0.001	98.10		
10	0.016±0.002	98.40		
12	0.015±0.007	98.50		

Table 4.14 DPPH absorbance of sample concentration for White Wine Grape Pomace extract

Sample Concentration (mg/ml)	Absorbance(λmax=517nm) Mean ± SD	% IA	IC_{50} (mg/l)	A_{AR}
1	0.612±0.057	39.10	1.49	0.67
2	0.389±0.032	61.12		
4	0.289±0.050	71.11		
6	0.205±0.002	79.56		
8	0.160±0.025	84.04		
10	0.139±0.008	86.14		
12	0.118±0.022	88.23		

A large number of studies have been conducted on RWGPE and have demonstrated excellent free radical scavenging, cardio protective properties and antiplatelet activity (Viuda-Martos *et al.*, 2005). In most cases, the activities of RWGPE are related to its anti-oxidative properties and are attributed mainly to the phenolic compounds. The data obtained reveal that the RWGPE phenolics are free-radical scavengers and primary antioxidants that react with free radicals. However, these results indicate that the phenolics present in the RWGPE have free radical-scavenging activities that are more significant than those present in the WWGPE, because of that the red grape pomace contains phenolic substances that embrace many classes of compounds, ranging from phenolic acids, colored anthocyanins and simple flavonoids to complex flavonoids (Kammerer *et al*., 2005). The activity of the extracts is attributed to their hydrogen-donating ability (Spigno *et al*., 2007). The higher activity of the RWGPE and WWGPE can be attributed to a more elevated concentration of TPC and antioxidants. The data obtained reveal that the extracts are free-radical inhibitors and primary antioxidants that react with free radicals. The IC_{50} value, defined as the concentration of extract required for 50% scavenging of DPPH or hydroxyl radicals under the experimental conditions employed, is a parameter widely used to measure free radical-scavenging activity; a smaller IC_{50} value corresponds to a higher antioxidant activity. The IC_{50} (mg/ml) values obtained for the samples submitted to the DPPH assay ranged from 1 to

12 mg/ml. The lowest IC_{50} values found were for RWGPE (1.24 mg/ml), which was also the richest in phenolics than WWGPE (1.49 mg/ml), exhibited moderate activity and control (2.67 mg/ml). These findings were lower than those described by Bravo(1998), who found that the DPPH (IC_{50}) free radical-scavenging activities of grape seeds from a variety of cultivars ('Merlot', 'Cabernet', 'Cinsault', 'Papaz Karasi', 'Ada Karasi', 'Hamburg Muscat', 'Alphonso Lavallee', 'Okuzgozu', 'Bogazkere', 'Senso' and 'Kalecik Karasi') cultivated in Turkey ranged from 2.71 mg/mL to 4.62 mg/ml, with an average value of 3.31 mg/ml.

Antiradical capacity (A_{AR}) is defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% and is expressed as $1/IC_{50}$. A high value of A_{AR} refers to high Antioxidant activity. The highest value of A_{AR} was found in the RWGPE (0.806), which was also the richest in phenolics, whereas WWGPE (0.671) exhibited the weakest activity. The larger the A_{AR}, the more efficient the antioxidant, as can be found in the RWGPE ($A_{A\ R}$= 0.806) more than in the WWGPE (A_{AR} = 0.671). Cruz *et al*.(2004) state that grape pomace presents a high antiradical activity (A_{AR}=0.71); this more coincides with this research findings for RWGPE and WWGPE. Sharp increases in radical scavenging activity (% inhibition) with an increase in the concentration (Figure 4.17) of extracts were observed up to 12 mg/ml concentration in RWGPE, WWGPE and control. As depicted in Figure 4.17 at this concentration, the RWGPE showed significantly higher activity (98.50%) than the ascorbic acid (96.27%) and WWGPE (88.23%). On the other hand, the higher activity of RWGPE than WWGPE can be attributed to a more elevated concentration of the TPC and antioxidants. The scavenging effect of extracts on the DPPH radical decreased in the order of RWGPE > WWGPE > Ascorbic acid (Figure 4.17).

The results showed that, although all the samples have noticeable effect on DPPH radical, RWGPE have sustainable hydrogen donating and radical scavenging ability than WWGPE and Ascorbic acid. Interestingly, RWGPE, which exhibited the highest content of total phenolic, total flavonoid, registered the highest DPPH radical scavenging potential compared to the standard ascorbic acid as well as WWGPE .And also WWGPE shows relatively lower than ascorbic acid (Figure 4.17). Different extraction condition may be the origin of the variance between the values; Cruz *et al*.(2004) have recently published lower values related to total antioxidant capacity for red wines from two Portuguese Appellations of Origin measured by DPPH with values ranging between 68.28% and 74.48 % having worked with 0.1 mL sample. On the other

hand, concerning the solvent used in the extraction step, Rockenbach *et al*. (2012) have worked with pomace (skins or seeds) from different Brazilian red grapes extracted by contact with an acidified mixture using methanol instead of ethanol. Their values were in average 85% to 92% of dry pomace for skins or seed extracts, respectively. The results in this study are comparable having worked with different extractive mixture, and moreover under extraction conditions safer and environmentally friendly as methanol is replaced by ethanol in this case.

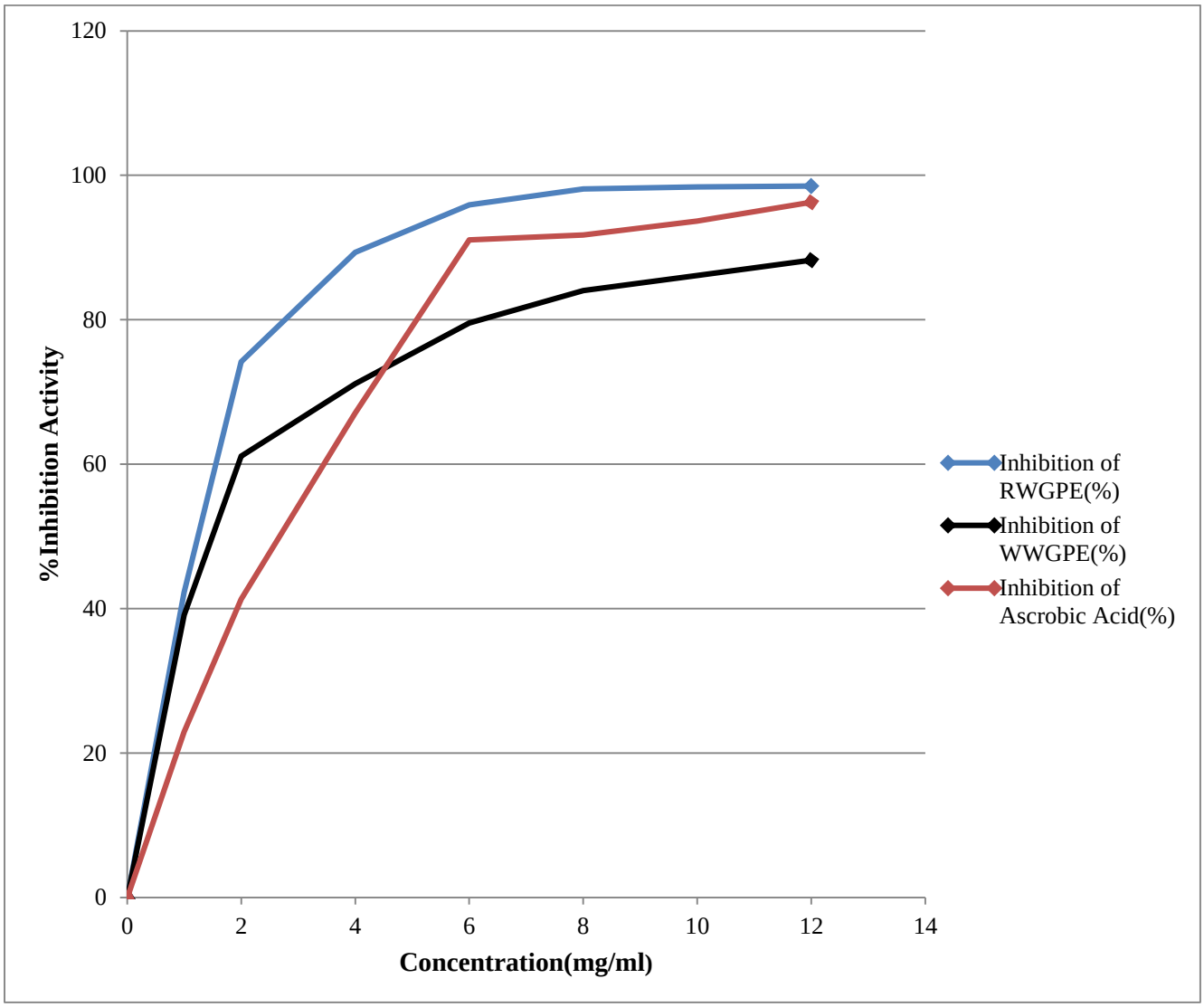


Figure 4.17 Comparison of percent DPPH free radical inhibition activities of ascorbic acid, Red and White Wine grape pomace extracts

4.3.6 Preservative Effect of Grape Pomace Extracts on Soybean Oil

Peroxide value, during storage of soy bean oil at 65°C were investigated as preservative effect both RWGPE and WWGPE extracts was studied on refined Soybean edible oils. The potential preservative effects of the extracts were evaluated based on the chemical analysis of peroxide value. Peroxide Value which indicates the deterioration of the model foods, edible oils, for this case. Increases of peroxide values are the best predictors of fat deterioration, which could be used to monitor the extent of oil spoilage.

The oxidation rate of soy bean oil was reduced by adding of RWGPE and WWGPE extracts. Peroxide value of oil decrease with RWGPE and WWGPE extract increasing its concentration in treated soybean oil Table 4.15 and 4.16 for RWGPE and WWGPE. RWGPE and WWGPE at 400ppm, 800ppm, 1200ppm and 1600ppm concentration had the higher peroxide value than BHT which shows lower inhibition oxidation than of BHT. For RWGPE and WWGPE extract (400,800,1200, and 1600 ppm) peroxide value of soy bean oil at 65°C increases and more higher than BHT (200ppm) for both RWGPE and WWGPE but RWGPE had lower peroxide value than WWGPE this due to RWGPE had higher antioxidant activity than WWGPE .

These results contradicts the findings of Duh and Yen,(1997) who observed that methanolic extracts of peanut hulls (used at 480 and 1200 ppm) inhibited peroxidation of both soy bean and peanut oils more efficiently than did BHA and BHT used at 200 ppm. Phenolic compounds are known to act as antioxidants not only due to their ability to donate hydrogen or electron but also attributed to their stable radical intermediates, which prevent the oxidation of various food ingredients particularly fatty acids (Makris *et al*., 2007). Development of rancidity in soy bean oil was affected by temperature and storage time.

A gradual increase in peroxide value of treated soy bean oil was observed during storage for 7 day at 65°C. The peroxide value of control sample increased from 1.25 to 21.51 after 4 days of storage at 65°C for RWGPE treated soybean oil. These concentrations does not extended the induction period at 400ppm and 800ppm to reach a peroxide value of 20 meq/kg in soybean oil under tested conditions (65°C) over 4 days but at concentration of 1200ppm and 1600ppm extends for 5 days and BHT 5 days similar but lower in the value of peroxide value .

However, there was distinct difference between synthetic antioxidants (BHT) and RWGPE as well as for WWGPE extracts in inhibition of soy bean oil peroxidation or peroxide value. This

due to it was visually observed that extracts did not well solubilize into the oil but formed a reddish layer on the glass at the interface oil/air. This work is not in agreement with other works which found a certain (not always high) protection of oil oxidation by different natural extracts (Bandonienė *et al.*, 2000 ; Sáyago-Ayerdi *et al*., 2009), which found that methanolic extract of citrus peel exhibited very strong antioxidant activity, which was almost equal to synthetic antioxidant (BHT). Wine Grape Pomace (WGP) has demonstrated several benefits in food applications, including inhibition of toxic oxidation product formation, maintenance of nutritional quality, and prevention of rancidity in lipid systems, and extension of food product shelf life as shown from the above table. For example, WGP extract prevents the secondary oxidation products formation in sunflower oil and the antioxidant effect is stronger than that of adding tocopherols in soybean oil. Schlesier *et al*.(2002) developed WGP fortified corn chips that obtained lower peroxide value after storage. In the seafood and meat industry, flavanol oligomers from WGP are the most potent oxidation inhibitors for emulsions in frozen fish muscles and increased lipid stability in chicken breast.

Table 4.15 Effect of BHT and Red Wine grape pomace extracts on peroxide value (meq/kg) of soy bean oil stored at 65^{o}C for 7 days

Storage day	control	BHT 200ppm	RWGPE			
			400ppm	800ppm	1200ppm	1600ppm
0	1.25	1.25	1.25	1.25	1.25	1.25
1	7.21	2.56	6.76	6.57	6.64	6.36
2	11.61	2.60	9.28	9.10	9.08	8.91
3	16.26	4.58	12.19	11.93	11.89	11.71
4	21.51	11.79	18.20	17.49	17.29	16.91
5	30.33	18.69	25.26	23.64	17.99	17.46
6	38.92	27.91	35.08	33.04	30.23	29.46
7	48.70	34.36	42.52	41.40	38.77	35.72

Table 4.16 Effect of BHT and White Wine grape pomace extracts on peroxide value (meq/kg) of soy bean oil stored at 65^{o}C for 7 days

Storage day	control	BHT 200ppm	WWGPE			
			400ppm	800ppm	1200ppm	1600ppm
0	1.25	1.25	1.25	1.25	1.25	1.25
1	7.21	2.56	6.98	6.79	6.52	6.50
2	11.61	2.60	10.83	10.55	10.44	9.41
3	16.26	4.58	14.19	13.93	12.88	12.75
4	21.51	11.79	20.00	19.88	19.29	19.01
5	30.33	18.69	28.66	28.34	27.99	27.66
6	38.92	27.91	37.08	36.88	35.90	35.46
7	48.70	34.36	42.52	41.40	38.77	35.72

Chapter Five

Conclusion and Recommendations

5.1 Conclusion

In this research, extraction and characterization of antioxidants and phenols from Red and White wine grape pomace were conducted. The effects of solvent ratio, extraction time and temperature on the yield of Red and White wine grape pomace extract have been studied. The results of the experiments conducted have been analyzed by employing Design-Expert 6.0.8, three-level-three-factor Central Composite Design (CCD) and Response Surface Methodology (RSM). In both varieties, extraction yield were significantly depending on the extraction conditions.

Generally, as extraction conditions (solvent ratio, time and temperature) increased from lower to center level, the extraction yield was increasing, but slightly increasing at some interval, as extraction conditions increased from center to high level individually. The extraction parameter of solvent ratio, extraction time and extraction temperature had significant (p<0.05) effect on the extract yield of Red and White wine grape pomace extracts.

It was confirmed that the factors, solvent ratio, extraction time and temperature, investigated in this study do affect the yield of extract from Red and White wine grape pomace. Statistical analysis showed that there are significant interactions between the factors and the response. The result in this study is in agreement with the previous studies stating that as ethanol ratio to water increase, the extraction time increases and extraction temperature, the extraction yield also increases in both RWGP and WWGP. A longer extraction time would give the bark and solvent better equilibrium and mass transfer. The solvent that gave the highest percentage yield of extract was 90:10 (v/v) ethanol to water. The optimum conditions that afforded the highest percentage yield of 52.26% in RWGP and WWGP were 90% ethanol solvent, 120 minutes of extraction time and 60 ^{o}C temperatures. The RWGP has a higher extract yield compared to WWGP.

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Physicochemical analysis of both extracts advocates it could serve as useful for food purposes and as a feedstock in many food and chemical industries and also form proximate composition analysis it was known both RWGP and WWGP has good nutritional value the results show that they were an important source of nutrients and compounds with functional properties so that they can be used for food fortification to add nutritional value and antioxidant effect on foods. Furthermore, the results of this study suggest that the flour produced from grape pomace, which is environmentally appropriate and easy to obtain, may be a potential food ingredient in the daily diet or as a nutritional supplement. The study also showed that the Red wine grape pomace is a good source rich in antioxidants and better in terms of extract yield and total phenolic content, total flavonoid content, total anthocyanin content and antioxidant activity by percentage of inhibition DPPH activity including preservative effect against oxidation on soybean oil. The comparison of Red and White wine grape pomaces form Awash Wine Factor by-products with their respective pomaces provided evidence that pomaces are very rich sources of antioxidants, despite extraction during vinification. Grape pomace extracts still contained appreciable amounts of total phenolic, total flavonoid and total anthocyanins. The quantitative distribution of (poly) phenols in grape pomaces showed significant differences through varieties that are from red wine grape pomace and white wine grape pomace.

In addition, Red wine grape pomace was shown to contain particularly high amounts of total phenolic, total flavonoid and total anthocyanin and thus an important antioxidant potential compared to White wine grape pomace because of this it shows higher antioxidant activity that was measured by ability to reduce free radical scavenging method. RWGPE also had the higher preservative effect on soybean oil by preliminary test by measuring peroxide value.

5.2 Recommendations

Given the demonstrated possibility of extracting antioxidants from Ethiopian Red and White wine grape pomaces *(Vitisvinifera L.)* waste, further works are still necessary. Future researches that would be complementary to the present study aiming at investigation of the process are, therefore, strongly recommended to put due focus on the following points:

- Antioxidants are sensitive in temperature, heat metal and oxygen, for better potent of antioxidant activity, future studies should focus on working in an inert atmosphere and away from light, would greatly prevent oxidation of polyphenols, and addition of antioxidant compounds such as ascorbic acid and SO_2 has been proposed and also freeze drying, with taking a care that they should be dried away from oxidation facilitating factors like heat, presence of oxygen and metal ions since in this study both fresh grape pomaces only frozen transported to laboratory and also both wine grape pomace were dried in the oven.
- Considering the large amount of by-products generated by the organic grape wine industry in Ethiopia, grape pomace extracts, grape skin flour and grape pomace flour represent promising materials for food companies in terms of product development and/or enrichment of different food/beverage formulations thus, the use of ethanol for recovering antioxidants from both Red and White wine grape pomace is very appealing because of its low cost which allows its use in the food industry and reduces the cost of the process so in our country emphasis should give towards this natural antioxidants which are safe, effective, easy to use and renewable starting from small house hold food processer.
- Quantitative analysis of phytocompounds (antioxidants) in ethanolic /water extracts by spectrophotometer analysis suggested that total phenolic content, total flavonoid content and total anthocyanin were present at the highest concentrations in both extracts special in RWGP, are good candidates for further development as nutraceutical supplements or antioxidant remedies so future studies should focus on the assessments of economic benefits and *in vivo* activities of these extracts before their commercial exploitation.
- It is suggested, that further studies should be conducted on this residue, to evaluate the presence of other bioactive compounds, including the evaluation of their antioxidant activity of the phenolic compounds and fatty acid composition in the seeds of this residue.

- Future researches should pay attention to innovative food applications into meat, fish, cereal, fruit-based and dairy products, with a focus on the type of recovered ingredient, and dosage level achieved by the application of this wine grape pomaces extract.
- The optimal storage conditions and shelf-life of extracts as a function of antioxidant activity has to be investigated and established in the future.
- Extracts/fractions from wine grape pomaces indicated antioxidant capacities in different testing systems, to determine which constituent(s) is/are the most important active components in different extracts/ fractions, HPLC (Higher Performance Liquid Chromatograph) should use for both qualitative and quantitative analyses in the future study related to Ethiopian wine making byproducts and in order to determine the phenol content and composition of the investigated extracts more precisely.
- Further work should be done to identify and characterize more inherent phytochemicals from different grape extracts and to evaluate their *in vivo* antioxidant potential and further work must performed to describe the antimicrobial activities in more detail and their application to obtain the active edible film.
- In the future, the extraction methods of polyphenols from grape should be improved, and the by-products of wine industry should be utilized effectively. The crude extracts from grape could be used as diet supplements for health-protection after defining the levels or limits to make sure the dose is safe for health.

References

Alonso, E., Bourzeix, M., & Revilla, E. (1991). Suitability of water/ethanol mixtures for the extraction of catechins and proanthocyanidins from Vitis vinifera seeds contained in a winery by-product. Seed Science & Technology.

Angshuman,and Chiranjit,B. (2012). *Extraction of polyphenols from dried tea leaves.* International Journal of Scientific and Engineering Research.

AOAC. (2002). *Official methods of analysis* (17 ed.). (M. Gaithersburg, Ed.) USA: Association of Official Analytical Chemists.

Atanackovic, M.,Petrovic A.,Jovic S.,Bukarica.,L.G Bursac M.,and Cvejic.J. (2012). *Inflence of wine making techniques on the resverstrol content,total phenolic content and antioxidant potential of red wines* (Vol. 131). food chemistry.

Bandoniené, D., Pukalskas, A., Venskutonis, P.R., & Gruzdiené D. (2000). Preliminaryscreening of antioxidant activity of some plant extracts in rapeseed oil. *Food Research*, 785-791.

Baş, D., & Boyacı, İ. H. (2007). Modeling and optimization I: Usability of response surface methodology. *Journal of Food Engineering, 78*(3), 836-845.

Bazykina, N.I., Nikolaevskii, A.N., Filippenko, T.A., & Kaloerova, V.G. (2002). Optimization of conditions for the extraction of natural antioxidants from raw plant materials. *Pharmaceutical Chemistry Journal, 36*(2), 100-103.

Boussetta, N., & Vorobiev, E. (2014). *Extraction of valuable biocompounds assisted by high voltage electrical discharges.* Comptes Rendus Chimie, .

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutrition reviews. *56*(11), pp. 317-333.

Cacace, J.E., & Mazza, G. (2003). Optimization of extraction of anthocyanins from black currants with aqueous ethanol. *Journal of Food Science, 68*(1), 240-248.

Castañeda-Ovando, A., Pacheco-Hernández, M. d. L., Páez-Hernández, M. E., Rodríguez, J. A., & Galán-Vidal, C. A. (2009). Chemical studies of anthocyanins: A review. Food Chemistry, 113(4), 859-871. *food chemistry, 859-871*(113), pp. 859-871.

Chamorro, S., Viveros, A., Alvarez, I., Vega, E., & Brenes, A. (2012). Changes in polyphenol and polysaccharide content of grape seed extract and grape pomace after enzymatic treatment. *133*, pp. 308-314.

Cruz, J.M., Domínguez, H., & Parajó, J.C. (2004). Assessment of the production of antioxidants from winemaking waste solids. *Journal of Agricultural and Food Chemistry, 24*, 5612-5620.

Duh, P.-D., and Yen, G.-C. (1997). Antioxidant Efficacy of Methanolic Extracts of Peanut Hulls in Soybean and Peanut Oils. *Journal of the American Chemists' Oil Society, 74*(6), 745-750.

El Darra, N., Grimi, N., Vorobiev, E., Louka, N., & Maroun, R. (2013). . *Extraction of polyphenols from red grape pomace assisted by pulsed ohmic heating.* (Vol. 6). Food and Bioprocess Technology.

El Gharras, H. (2009). *Polyphenols: food sources, properties and applications.* (Vol. 44). a review. International Journal of Food Science & Technology,.

En-Qin, X., Gui-Fang, D., Ya-Jun, G., & Hua-Bin, L. (2010). *Biological Activities of Polyphenols from Grapes.* (Vol. 11). International Journal of Molecular Sciences.

FAO.(2016, november 16). *Agribusiness Handbook, Grapes wines.* Retrieved november 16, 2016, from from http://www.fao.org/.

Fernández, K. V. (2015). An enzymatic extraction of proanthocyanidins from País grape seeds and skins. . In *Food chemistry* (Vol. 168, pp. 7-13).

Fontana, A. R., Antoniolli, A., & Bottini, R. (2013). Grape Pomace as a Sustainable Source of Bioactive Compounds: Extraction Characterization, and Biotechnological Applications of Phenolics. *Journal of Agriculture and Food Chemistry, 61*(38), 8987-9003.

Gonzalez-Montelongo, R. M., Gloria Lobo, G. and Gonzalez, M. (2010). Antioxidant activity in banana peel extracts: Testing extraction conditions and related bioactive compounds. *Food Chemistry, 119*, 1030-1039.

Goupil, P., Benouaret, R., Charrier, O., Ter Halle, A., Richard, C., Eyheraguibel, B., Thiery, D., & Ledoigt, G. (2012). Grape marc extract acts as elicitor of plant defense responses. *Ecotoxicology,, 21*(5), 1541-1549.

Guendez, R., Kallithraka, S., Makris, D. P., & Kefalas, P. (2005). Determination of low molecular weight polyphenolic constituents in grape (Vitis vinifera sp.) seed extracts: Correlation with antiradical activity. Food Chemistry, 89(1), 1-9.

Gülçin, İ. (2010). Antioxidant properties of resveratrol: A structure–activity insight. Innovative Food Science & Emerging Technologies. *11*(1), 210-218.

Gülçin, I. (2012). Antioxidant activity of food constituents: An overview. Archives of Toxicology. *86*(3), 345-391.

Hadolin, M., Hraš, A.R., Bauman, D., & Knez, Z. (2004). Isolation and concentration of natural antioxidants with high-pressure extraction. *Innovative Food Science and Emerging Technologies, 5*, 245-248.

Hagerman, A. E. (2002). Tannin handbook O. O. .

Hollmann, P.C.H., & Katan,M.B. (1999). Dietary flavonoids: intake, health effects and bioavailabity. In *Food and Chemical Toxicology* (Vol. 37, pp. 937-942).

Huang, D., Boxin, O. U., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *53*(6), 1841-1856.

Ignat, I., Volf, I., & Popa, V. I. (2011). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry,, 126*(4), 1821-1835.

Juntachote, T., Berghofer, E., Bauer, F., & Siebenhandl, S. (2006). The application of response surface methodology to the production of phenolic extracts of lemon grass, galangal, holy basil and rosemary. *International Journal of Food Science and Technol*, 121-133.

Kammerer, D., Claus, A., Schieber, A., & Carle, R. (2005). A Novel Process for the Recovery of Polyphenols from Grape (Vitis vinifera L.) Pomace. . *Journal of Food Science,, 70*(2), 157-163.

Kearney, J. (2010). *Food consumption trends and drivers.* (Vol. 365). Philosophical transactions of the royal society B: biological sciences, .

Kim, S-Y., Jeong, S-M., Park, W-P., Nam, K.C., Ahn, D.U., & Lee, S-C. (2006). *Effect of heating conditions of grape seeds on the antioxidant activity of grape seed extracts* (97 ed.).

Kirby, A.J. and Schmidt, R.J. (2004). The antioxidant activity of Chinese herbs for eczema and of placebo herbs. . *Ethnopharmacol., 56*, 103-108.

Konczak, I. (2004). *Anthocyanins—more than nature's colours.* (Vol. 5). BioMed Research International, .

Lapornik, B., Prošek, M., & Golc Wondra, A. (2005). Comparison of extracts prepared from plant by-products using different solvents and extraction time. *Journal of Food Engineering,, 71*(2), 214-222.

Laufenberg, G., Kunz, B., & Nystroem, M. (2003). Transformation of vegetable waste into value added products: the upgrading concept; practical implementations. *Bioresource Technology,, 87*(2), 167-198.

Lorrain, B., Ky, I., Pechamat, L., & Teissedre, P.L. (2013). Evolution of Analysis of Polyhenols from Grapes, Wines, and Extracts. Molecules, 18(1), 1076- 1100. *18*(1), 1076-1100.

Lu, Y., & Yeap Foo, L. (1999). The polyphenol constituents of grape pomace. In *Food Chemistry*, (Vol. 65, pp. 1-8).

Luque de Castro, M., & Priego-Capote, F. (2007). Ultrasound assistance to liquid–liquid extraction. *Analytica Chimica Acta, 518*(1), pp. 2-9.

Magalhães, L. M., Segundo, M. A., Reis, S., & Lima, J. L. F. C. (2008). Methodological aspects about in vitro evaluation of antioxidant properties. *Anal Chim Acta, 613*(1), 1-19.

Makris, D. P., Boskou, G., & Andrikopoulos, N. K.(2007). Polyphenolic content and in vitro antioxidant characteristics of wine industry and other agrifood solid waste extracts. *Journal of Food Composition and Analysis,, 20*(2), 125-132.

Mendes, J. A. S., Prozil, S. O., Evtuguin, D. V., & Lopes, L. P. C. (2013). Towards comprehensive utilization of winemaking residues: Characterization of grape skins from red grape pomaces of variety Touriga Nacional. Industrial Crops and Products, .

Montgomery, D. (2005). *Design and Analysis of Experiments, 5th edition.* New York: John Wileyand Sons.

Moure, A., Cruz, J. M., Franco, D., Dominguez, J. M., Sineiro, J., Dominguez, H., Nunez, M. J. and Parajo, J. C. (2001). Natural antioxidants from residual sources. *Food Chemistry, 72*, 145-171.

OIV., (2012). *Organisation Internationale de la Vigne et du Vin.* . Retrieved september 16, 2016, from http://WWW.oiv.int/oiv/info/es.

OIV.,(2014, october 23). *Organisation Internationale de la Vigne et du Vin.* . Retrieved december 28, 2016, from http://www.oiv.int/oiv/info/es_OIV_Press_Conference.

Omoba, O. S., Obafaye, R. O., Salawu, S. O., Boligon, A. A. and Athayde, M. L. (2015). *HPLC-DAD Phenolic Characterization and Antioxidant Activities of Ripe and Unripe Sweet Orange Peels* (Vol. 4).

Osborne, D.R., and Voogt, P. (1978). The Analysis of nutrients in foods. *Official methods*, 6.2, 6.3.

Pekić, B., Kovač, V., Alonso, E., & Revilla, E. (1998). *Study of the extraction of proanthocyanidins from grape seeds* (Vol. 61). Food Chemistry.

Pinelo, M., Del Fabbro, P., Marzocco, L., Nunez, M.J. & Vicoli, M.C. (2005). Optimization of continuous phenol extraction from Vitis vinifera byproducts. *Food Chemistry, 92,* pp. 109-117.

Ping, L., Pizzi, A., Guo, Z. D., & Brosse, N. (2011). *Condensed tannins extraction from grape pomace: Characterization and utilization as wood adhesives for wood particleboard.* Industrial Crops and Products,.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agriculture and Food Chemistry, 53*(10), 4290-4302.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). *Structure-antioxidant activity relationships of flavonoids and phenolic acids.* Free Radical Biology and Medicine.

Rockenbach, I. I., Jungfer, E., Ritter, C., Santiago-Schübel, B., Thiele, B., Fett R., & Galensa, R. (2012). *Characterization of flavan-3-ols in seeds of grape pomace by CE, HPLC-DAD-MSn and LC-ESI-FTICR-MS.* Food Research International.

Sáyago-Ayerdi, S. G., Brenes, A., & Goñi, I. (2009). *Effect of grape antioxidant dietary fiber on the lipid oxidation of raw and cooked chicken hamburgers.* LWT - Food Science and Technology,.

Schlesier, K., Harwat, M., Böhm, V., & Bitsch, R. (2002). *Assessment of antioxidant activity by using different in vitro methods.* Free Radical Research.

Shirahigue, L. D., Plata-Oviedo, M., de Alencar,S. M., d'Arce, M. A. B. R., de Souza Vieira, T. M. (2010). Wine industry residue asantioxidant in cooked chicken meat. *International Journal of Food Science &Technology, 45(5), 863-870., 45*(5), 863-870.

Singh, S. and Immanuel, G. (2014). *Extraction of Antioxidants from Fruit Peels and its Utilization in Paneer.* (F. P. Technol, Ed.) Food Processing & Technology.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods In Enzymology, 299, 152-178.*(299), 152-178.

Spigno, G., Tramelli, L., & De Faveri, D.M. (2007). solvent on concentration and antioxidant activity of grape marc phenolics. *Journal of Food Engineering, 81,* 200-208.

Sukhdev, S. H., Suman Preet, S. K., Gennaro , L. and Dev, D. R. (2008). Extraction technologies formedicinal and Aromatic plants. *International Centre for Science and High Technology,*.

Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D. A. (2014). Natural Bioactive Compounds from Winery By-Products as Health Promoters. *A Review. International Journal of Molecular Sciences, 15*(9), 15638-15678.

Viuda-Martos, M., Ruiz Navajas, Y., Sánchez Zapata, E., Fernández-López, J., & Pérez-Álvarez, J. A. (2010). Antioxidant activity of essential oils of five spice plants widely used in a Mediterranean diet. *Flavour and Fragrance Journal, 25*(1), 13-19.

Wang, L., & Weller, C. L. (2006). *Recent advances in extraction of nutraceuticals from plants* (Vol. 17). Trends in Food Science & Technology.

Wells, M. J. (2003). *Principles of extraction and the extraction of semivolatile organics from liquids. Sample Preparation Techniques in Analytical Chemistry, .*

Wijngaard, H., Hossain, M. B., Rai, D. K., & Brunton, N. (2012). *Techniques to extract bioactive compounds from food by-products of plant origin.* (Vol. 46). Food Research International, 46(2).

Yilmaz, Y., & Toledo, R.T. (2006). Oxygen radical absorbance capacities of grape/wine industry byproducts and effect of solvent type on extraction of grape seed polyphenols. *Journal of Food Composition and Analysis, 19*, 41-44.

Yu, J. M., & Ahmedna, M. (2013). . Functional components of grape pomace: their composition, biological properties and potential applications. *International Journal of Food Science and Technology, 48*(2), 221-237.

Zhang, H. Y. and Wang, L. F. (2005). Solvent effects are important in elucidating radical scavenging mechanisms of antioxidants. A case study on genistein. *J Biomol Struct Dyn, 2*, 483-486.

Appendixes

Appendix A: Raw data for characterization of antioxidants (RWGPE and WWGPE) by UV-Spectrophotometer

A. For Total Phenolic Content

Table A1 Gallic acid standard solution preparation and corresponding absorbance

Gallic acid(μL)	Methanol (μL)	Concentration (mg/ml)	FC (ml)	Na_2NO_3 (ml)	Distil water (ml)	absorbance	Absorbance Mean ± SD
0	1000	0	1	1	7	0.000	
20	980	1.0	1	1	7	0.88	0.95±0.242
20	980	1.0	1	1	7	0.75	
20	980	1.0	1	1	7	1.22	
40	960	2.0	1	1	7	1.203	1.072±0.102
40	960	2.0	1	1	7	1.003	
40	960	2.0	1	1	7	1.190	
80	920	4.0	1	1	7	1.693	1.749±0.072
80	920	4.0	1	1	7	1.723	
80	920	4.0	1	1	7	1.831	
120	880	6.0	1	1	7	2.102	2.092±0.038
120	880	6.0	1	1	7	2.050	
120	880	6.0	1	1	7	2.124	
160	840	8.0	1	1	7	2.753	2.831±0.071
160	840	8.0	1	1	7	2.893	
160	840	8.0	1	1	7	2.847	
200	800	10.0	1	1	7	3.212	3.176±0.102
200	800	10.0	1	1	7	3.256	
200	800	10.0	1	1	7	3.060	
240	760	12.0	1	1	7	3.378	3.366±0.061
240	760	12.0	1	1	7	3.299	
240	760	12.0	1	1	7	3.421	

Table A2 Sample solution preparation and corresponding absorbance for RWGPE

Gallic acid(μL)	Methanol (μL)	Concentration (mg/ml)	FC (ml)	Na_2NO_3 (ml)	Distil water (ml)	absorbance	Absorbance Mean ± SD
20	980	1.0	1	1	7	0.263	0.195±0.071
20	980	1.0	1	1	7	0.200	
20	980	1.0	1	1	7	0.121	
40	960	2.0	1	1	7	0.502	0.424±0.106
40	960	2.0	1	1	7	0.302	
40	960	2.0	1	1	7	0.467	
80	920	4.0	1	1	7	0.655	0.685±0.071
80	920	4.0	1	1	7	0.633	
80	920	4.0	1	1	7	0.767	
120	880	6.0	1	1	7	0.877	0.893±0.040
120	880	6.0	1	1	7	0.855	
120	880	6.0	1	1	7	0.947	
160	840	8.0	1	1	7	1.188	1.151±0.102
160	840	8.0	1	1	7	1.230	
160	840	8.0	1	1	7	1.035	
200	800	10.0	1	1	7	1.008	1.182±0.102
200	800	10.0	1	1	7	1.230	
200	800	10.0	1	1	7	1.035	
240	760	12.0	1	1	7	1.102	1.230±0.118
240	760	12.0	1	1	7	1.250	
240	760	12.0	1	1	7	1.337	

Table A3 Sample solution preparation and corresponding absorbance for WWGPE

Gallic acid(μL)	Methanol (μL)	Concentration (mg/ml)	FC (ml)	Na_2NO_3 (ml)	Distil water (ml)	absorbance	Absorbance Mean ± SD
20	980	1.0	1	1	7	0.135	0.123±0.014
20	980	1.0	1	1	7	0.127	
20	980	1.0	1	1	7	0.107	
40	960	2.0	1	1	7	0.456	0.389±0.089
40	960	2.0	1	1	7	0.423	
40	960	2.0	1	1	7	0.288	
80	920	4.0	1	1	7	0.702	0.623±0.101
80	920	4.0	1	1	7	0.658	
80	920	4.0	1	1	7	0.509	
120	880	6.0	1	1	7	0.843	0.789±0.045
120	880	6.0	1	1	7	0.753	
120	880	6.0	1	1	7	0.798	
160	840	8.0	1	1	7	1.203	1.089±0.101
160	840	8.0	1	1	7	1.056	
160	840	8.0	1	1	7	1.008	
200	800	10.0	1	1	7	1.250	1.105±0.125
200	800	10.0	1	1	7	1.032	
200	800	10.0	1	1	7	1.032	
240	760	12.0	1	1	7	1.210	1.149±0.053
240	760	12.0	1	1	7	1.125	
240	760	12.0	1	1	7	1.112	

B. For Total Flavonoid Determination

Table A4 Catechin standard solution preparation and the corresponding absorbance

Catechin (µL)	Methanol (µL)	Concentration (mg/ml)	$NaNO_2$ (µL)	$AlCl_3.6H_2O$ (µL)	NaOH (ml)	absorbance	Absorbance Mean ± SD
0	1000	0	75	150	0.5	0.000	0
100	900	0.05	75	150	0.5	0.321	0.234±0.123
100	900	0.05	75	150	0.5	0.147	
200	800	0.10	75	150	0.5	0.563	0.448±0.162
200	800	0.10	75	150	0.5	0.333	
300	700	0.15	75	150	0.5	0.672	0.658±0.019
300	700	0.15	75	150	0.5	0.644	
400	600	0.20	75	150	0.5	0.799	0.869±0.098
400	600	0.20	75	150	0.5	0.939	
500	500	0.25	75	150	0.5	1.166	1.083±0.117
500	500	0.25	75	150	0.5	1.000	
600	400	0.30	75	150	0.5	1.230	1.294±0.090
600	400	0.30	75	150	0.5	1.358	
700	300	0.35	75	150	0.5	1.452	1.504±0.073
700	300	0.35	75	150	0.5	1.556	
800	200	0.40	75	150	0.5	1.893	1.734±0.224
800	200	0.40	75	150	0.5	1.575	
900	100	0.45	75	150	0.5	1.888	1.954±0.093
900	100	0.45	75	150	0.5	2.020	
1000	0	0.5	75	150	0.5	2.210	2.154±0.079
1000	0	0.5	75	150	0.5	2.098	

C. For Antioxidant activity determination

Table A5 Ascorbic acid standard curve preparation

Ascorbic acid (µL)	Methanol (µL)	Concentration (mg/ml)	DPPH (ml)	Absorbance	Absorbance Mean ± SD
0	1000	0	4	1.003	
20	980	1.0	4	0.802	0.773±0.041
20	980	1.0	4	0.744	
40	960	2.0	4	0.542	0.588±0.065
40	960	2.0	4	0.634	
80	920	4.0	4	0.380	0.350±0.042
80	920	4.0	4	0.320	
120	880	6.0	4	0.042	0.092±0.070
120	880	6.0	4	0.141	
160	840	8.0	4	0.095	0.085±0.014
160	840	8.0	4	0.075	
200	800	10.0	4	0.552	0.063±0.340
200	800	10.0	4	0.070	
240	760	12.0	4	0.041	0.037±0.005
240	760	12.0	4	0.330	

Table A6 Preparation of DPPH absorbance for RWGPE

sample (µL)	Methanol (µL)	Concentration (mg/ml)	DPPH (ml)	Absorbance	Absorbance Mean ± SD
20	980	1.0	4	0.620	0.579±0.057
20	980	1.0	4	0.538	
40	960	2.0	4	0.280	0.259±0.029
40	960	2.0	4	0.238	
80	920	4.0	4	0.106	0.107±0.001
80	920	4.0	4	0.108	
120	880	6.0	4	0.038	0.041±0.004
120	880	6.0	4	0.044	
160	840	8.0	4	0.020	0.019±0.001
160	840	8.0	4	0.018	
200	800	10.0	4	0.018	0.016±0.002
200	800	10.0	4	0.014	
240	760	12.0	4	0.020	0.015±0.007
240	760	12.0	4	0.010	

Table A7 Preparation of DPPH absorbance for WWGPE

sample (μL)	Methanol (μL)	Concentration (mg/ml)	DPPH (ml)	Absorbance	Absorbance Mean ± SD
20	980	1.0	4	0.653	0.612±0.057
20	980	1.0	4	0.571	
40	960	2.0	4	0.366	0.389±0.032
40	960	2.0	4	0.412	
80	920	4.0	4	0.253	0.289±0.050
80	920	4.0	4	0.325	
120	880	6.0	4	0.203	0.205±0.002
120	880	6.0	4	0.207	
160	840	8.0	4	0.142	0.160±0.025
160	840	8.0	4	0.178	
200	800	10.0	4	0.145	0.139±0.008
200	800	10.0	4	0.133	
240	760	12.0	4	0.102	0.118±0.022
240	760	12.0	4	0.134	

Appendix B: Diagrams and Photos

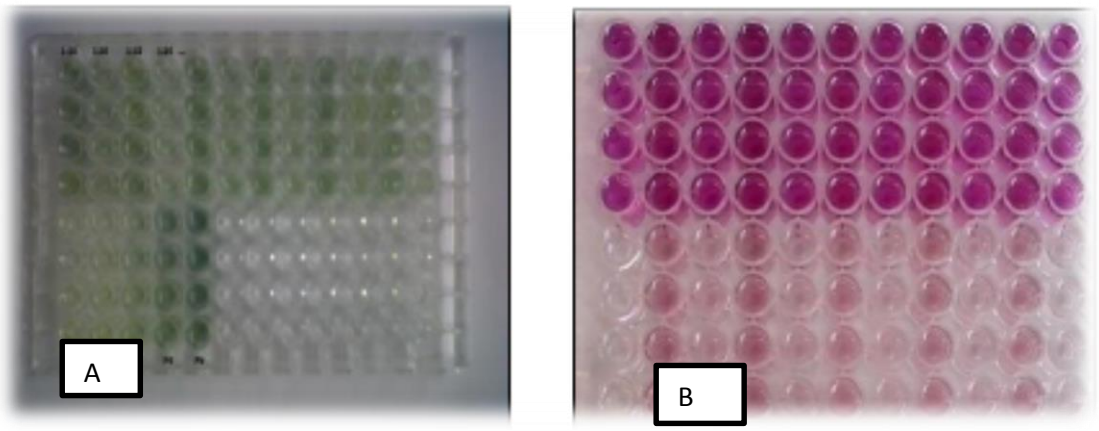

Figure B1 Examples of microplates used for Folin-Ciocalteu (A) and DPPH (B)

Figure B2 General Scheme for preparation of grape pomace extracts RWGPE and WWGPE

DECLARATION

By submitting this thesis I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof to the extent where it is explicitly stated otherwise, that reproduction and publication thereof by any party none other than Addis Ababa University's School of Chemical and Bio Engineering (Graduate study) will not infringe any third party rights and that I have not previously submitted it entirely or in part for obtaining any qualification.

Tamirat Endale Signature Date 19/June/2017