ULTRASOUND ASSISTED EXTRACTION OF PHENOLICS FROM GRAPE POMACE

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ABSTRACT

ULTRASOUND ASSISTED EXTRACTION OF PHENOLICS FROM GRAPE POMACE

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Grape pomace is a by-product of wineries. It is one of the most potent antioxidant sources due to its high phenolic content. In this thesis study, ultrasound assisted extraction of phenolic compounds from Merlot grape pomace has been studied. The effects of sonication time, subsequent extraction time in shaking water bath at 45°C and composition of the solvent on extraction efficiency and recovery of phenolics were studied by response surface methodology. Folin-Ciocalteu colorimetric method was used to analyze effects of process parameters on the total phenolic content of the extracts. The best recovery (47.2 mg gallic acid equivalents of total phenolics per g of dried grape pomace) was obtained using 30 % aqueous ethanol and applying 6 minutes of sonication followed by 12 minutes of shaking in water bath at 45°C.

Keywords: Grape pomace, total phenolics, ultrasonication, response surface methodology

ÖZ

FENOLİK MADDELERİN ÜZÜM CİBRESİNDEN ULTRASON DESTEKLİ ÖZÜTLENMESİ

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Ocak 2006, 69 sayfa

Üzüm cibresi şarap fabrikalarının yan ürünüdür. Cibre, yüksek fenolik madde içeriği nedeniyle önemli bir antioksidan kaynağı olarak kabul edilmektedir. Bu tez çalışmasında fenolik maddelerin Merlot türü üzüm cibresinden ultrason destekli ekstraksiyon koşulları araştırılmıştır. Ultrason süresi, çalkalamalı sıcak su banyosunda bekletme süresi (45°C) ve çözücü bileşiminin özütleme sürecine ve elde edilen fenolik madde miktarına etkisi yanıt yüzey yöntemiyle incelenmiştir. Parametrelerin etkisi ve özütün fenolik içeriği Folin ve Ciocalteu spektrofotometrik yöntemiyle gallik asit eşdeğeri olarak tayin edilmiştir. Özütte en yüksek fenolik içerik (kurutulmuş cibrenin gramı başına 47.2 mg gallik asit eşdeğeri toplam fenolik madde) çözücünün hacimce 30 % oranında etil alkol içerdiği, 6 dakika ultrason süresini müteakip 12 dakika 45°C sıcaklıkta çalkalamalı sıcak su banyosunda bekletildiği deney noktasında elde edilmiştir.

Anahtar Sözcükler: Üzüm cibresi, toplam fenolik madde, ultrason, yanıt yüzey yöntemi

To the memory of my uncle,

Kenan Kahraman

(1960-2003)

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LIST OF SYMBOLS AND ABBREVIATIONS

Edf	Pure error degree of freedom
ESS	Pure error sum of square
EtOH	Ethanol (Ethyl Alcohol)
F_{LOF}	F value for lack of fit
F_{MOD}	F value of statistical model
GAE	Gallic Acid Equivalent
Ldf	Lack of fit degree of freedom
LSS	Lack of fit sum of square
MSE	Mean square of pure error
MSL	Mean square of lack of fit
OFR	Oxygen free radicals
n_c	Number of factorial points
n_a	Number of star points
n_o	Number of factorial points
Rdf	Residual degree of freedom
ROS	Reactive oxygen species
RSM	Response surface methodology
RSS	Residual sum of square
SC	Solvent Composition
ST	Sonication Time
TEP	Total extractable polyphenolics
TCA	Trichloroacetic Acid
WBT	Water Bath Time

X_1	Parameter level of sonication time
X_2	Parameter level of water bath time
X_3	Parameter level of solvent composition
x_1	Coded level of sonication time
x_2	Coded level of water bath time
x_3	Coded level of solvent composition
α	General notation of coded level of star points

CHAPTER 1

INTRODUCTION

1.1 FREE RADICALS

A free radical can be defined as any atom or molecule that posses an unpaired electron. It can be anionic, cationic or neutral. Possessing an unpaired electron make free radicals highly reactive and unstable species. In biological and related fields, oxygen free radicals (OFRs) are the major free radical species of interest [Punchard & Kelly, 1996]. OFRs are part of a greater group of molecules called reactive oxygen species (ROS). This group of molecules includes hydrogen peroxide (H_2O_2), lipid peroxide (LOOH), singlet oxygen ($^1O_2$), hypochlorus acid (HOCl) and other N-Chloramine compounds. In addition to OFRs, carbonyl, thiyl and nitroxyl radicals are also important radical species. OFRs are potentially very toxic to cells. They can easily combine with other molecules within the cell such as enzymes, receptors and ion pumps due to their high reactive nature, and they cause oxidation directly, result in inactivating or inhibiting normal function of cells [Punchard & Kelly, 1996].

One of the most destructive effects of OFRs is the initiation of lipid peroxidation, resulting in destruction of celluar membranes, by generating a conjugate diene, which after rearrangement, will easily combine with oxygen to give a lipid peroxyl radical. This radical can abstract hydrogen from another polyunsaturated fatty acid (PUFA) to give a lipid hyperoxide and a new lipid radical that can repeat of the events. If these chain events are not terminated they will result in destruction of cell membranes, break down of compartmentalization and finally release of lysosomal enzymes and subsequent autolysis [Punchard & Kelly, 1996].

Such kind of chain reactions should be terminated before giving substantial damages on different parts of the cell. Termination can be achieved by chain breaking antioxidants.

OFRs and other free radical species can be generated by normal metabolic processes, such as the reduction of oxygen to water by the mitochondrial electron transport chain from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants and industrial chemicals [Punchard & Kelly, 1996; Bagchi & Puri, 1998]. On the other hand, it is known that biological processes are not 100 % efficient, and it is thought that from 1 % to 5 % of all oxygen used in metabolism can escape as free radical intermediates. Therefore, the generation of free radicals seems to be a part of cellular metabolism [Punchard & Kelly, 1996].

Free radicals can accelerate aging and can cause the development of various diseases including cancer, autoimmune, inflammatory, cardiovascular and neuro-degenerative (e.g. Alzheimer's disease, Parkinson's disease, multiple sclerosis, Downs syndrome) diseases [Aruoma, 2003; Bagchi & Puri, 1998].

On the other hand, oxidation is one of the most important problems in the food industry, especially in food preservation. Organic matter, due to its nature, has a strong tendency to react with oxygen and oxidize. Therefore, the spontaneous reaction of atmospheric oxygen with organic compounds results in a number of degradative effects on food products. In the food degradation, OFRs and ROS play critical roles. Any food will eventually oxidize even if it is stored at temperatures as low as - 70°C [Punchard & Kelly, 1996]. Therefore, degradation of food occurs, and unpleasant taste and odor develops. In order to prevent oxidation reactions and to extend the shelf life of product, a variety of antioxidants are used as food additives. Thus, it can be said that antioxidants play a central role in food quality and safety. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are powerful inhibitors for lipid peroxidation. However, long-term in vivo studies have been reported that they could promote tumor formation [Barlow, 1990]. The potential adverse effects of such synthetic antioxidants and increase in demand of natural sources have,

therefore, led research studies on antioxidants to concentrate not only on their functionality, but also on their sources, health aspects, and effectivenesses in prevention of certain diseases [Pszczola, 2001].

1.2 ANTIOXIDANTS

A substance which, when present at low concentrations with that of an oxidizable substrate, significantly delays or prevents oxidation of substrate (lipids including polyunsaturated fatty acids, proteins, carbohydrates, DNA) would be considered as an antioxidant [Aruoma, 2003]. The term food antioxidant is generally applied to those compounds that interrupt the free-radical chain reactions involved in lipid oxidation [Rossel & Kochhar, 1990]. Broadly speaking, antioxidants may be classified into five groups [Rossel & Kochhar, 1990] as follows:

1.2.1 Primary Antioxidants

They are also referred to as chain-breaking antioxidants. They have ability to react with lipid radicals to convert them to more stable products. They are mainly phenolic substances. More specifically, a molecule will be able to act as a primary antioxidant if it is able to donate a hydrogen atom rapidly to a lipid radical ($ROO^{\cdot}$) and if the radical derived from the antioxidant is more stable than the lipid radical, or is converted to more stable products [Punchard & Kelly, 1996]. Natural and synthetic tocopherols, alkyl gallates, butylated hydroxyanisole (BHA), butylated hydroxytolune (BHT), tertiary butyl hydroquinone (TBHQ) etc. belong to this group and function as electron donating agents. However, BHA and BHT are synthetic compounds, which are used as food preservatives, and their usage have been restricted in the United States since 1995, because they have been found suspicious structures of carcinogenesis [Barlow, 1990].

1.2.2 Oxygen Scavengers

Ascorbic acid (Vitamin C), ascorbyl palmite, erthorbic acid (d-isomer of ascorbic acid) and its sodium salt etc. belongs to this group of antioxidants. They can react with oxygen and can thus remove it in a closed system.

1.2.3 Secondary Antioxidants

They are also known as preventive antioxidants and they reduce the rate of chain initiation by a variety of mechanisms including compounds that bind metal ions, scavenge oxygen, decompose hydroperoxides to non-radical species, absorb UV radiation or deactivate singlet oxygen. Dilauryl thiopropionate and thiodiproponic acid, which function by decomposing the lipid hydroperoxides into stable end products, examples of this category.

1.2.4 Enzymic Antioxidants

This group of antioxidants function as either by removing dissolved/headspace oxygen or by removing highly oxidative species from food systems. Glucose oxidase, superoxide dismutase, catalase, glutathione peroxidase, etc. belong to this group of antioxidants.

1.2.5 Chelating Agents (Sequestrants)

Citric acid, amino acids ethylenediamine tetra acetic acid (EDTA) etc. chelate metallic ions such as copper and iron that promote lipid oxidation through a catalytic reaction. The chelates are sometimes referred to as synergists since they greatly increase the

action of phenolic antioxidants. Most of these synergists exhibit little or no activity when used alone, except amino acids, which can show antioxidant or pro-oxidant activity.

1.3 PHENOLICS in GRAPE

Phenolics contain multiple hydroxyl groups. They are hydrogen-donating antioxidants and singlet oxygen quenchers [Kandawasmi & Middleton, 1994]. They are also very powerful metal chelating agents. They can trap free radicals and break chain initiation reactions [Shi, Pohorly and Kakuda, 2003]. As a general categorization proposed by Shi et al. (2003) phenolic compounds in grape can be divided into two groups: phenolic acids (precursors of flavonoids) and flavonoids. The most common phenolic acids encountered in grape are cinnamic acids (coumeric, caffeic, ferulic, chlorogenic and neochlorogenic acids) and benzoic acids (p-hydroxybenzoic, protocathecuic, vanillic and gallic acids). Flavonoids in grape mainly consist of flavan-3-ols (catechin, epicatechin, their polymers and their ester forms with glucose), flavanones (the most common one being quercetin) and anthocyanins.

Phenols represent the third most abundant constituent in grapes after carbohydrates and fruit acids [Singleton, 1980]. In addition, the distribution of total extractable phenolics of fresh grape was indicated as about 10 % in pulp, 60-70 % in seeds, and 28-35 % in skin. The phenolic content of grape seeds may be in the range from 5 % to 8 % by weight [Shi et. al., 2003].

Although the flavonoids are specific for plastid containing plant cells, there is no evidence indicating participation of the flavonoids in the primary photosynthetic processes. Instead, there are evidences indicating the role of flavonoids in gene regulation [Havsteen, 2003].

Flavonoids have structural variations in carbon ring that identifies the different types, namely, flavonols, flavones, isoflavones, flavonones, flavonol and anthocynanins [Aruoma, 2003], as indicated in Figure-1.

The specificity and great diversity of flavonoids makes the taxonomical classification difficult. The reasons of the great variability of flavonoids are as follows [Havsteen, 2003]:

- Differences in the ring structure of aglycone and its state of oxidation/reduction,
- Differences in extent of hydroxylation of the aglycone and in the position of hydroxyl groups,
- Differences in the derivatisation of the hydroxyl groups, for example with methyl groups, carbohydrates or isoprenoids etc.

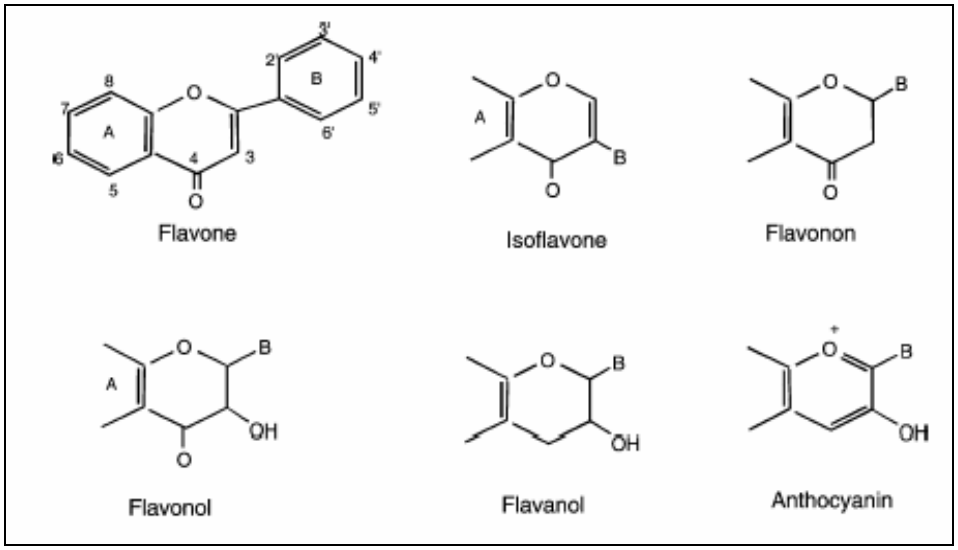

Figure-1: Molecular Skeletons of Major Flavonoids [adapted from Havsteen, 2000]

As has been stated, the phenolic compounds in grape are essentially all flavonoids and they consist of mainly flavan-3-ols, namely, catechin and epicatechin. If a third OH group is added to B ring, then epigallocatechin and catechin are formed. Furthermore, if a gallic acid is bonded to C3 position, then gallates are formed. The molecular structures of these compounds are indicated in Figure-2.

Oligomeric procyanidins (OPCs) and polymeric compounds (condensed tannins) are derived from these monomeric units. OPCs containing two monomers are called dimers and three monomers are called trimers. The dimeric and trimeric procyanidins are known as B-series and C-series respectively. Five different dimers (Procyanidin B1, B2, B3, B4 and B5) and two trimers (C1 and C2) were characterized from grape skin and seeds [Shi et. al., 2003]. The chemical structures of procyanidin dimers, trimers and tetramers are indicated in Figure-3, 4, and 5 respectively.

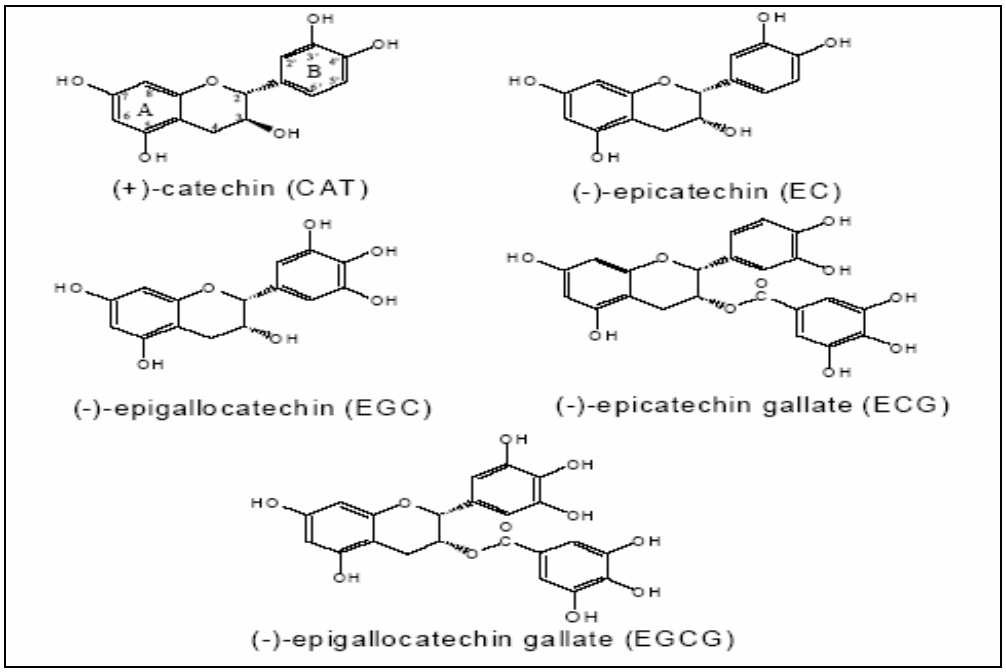

Figure-2: Monomeric Flavonoids and Derivatives [Adapted from Bors et al.]

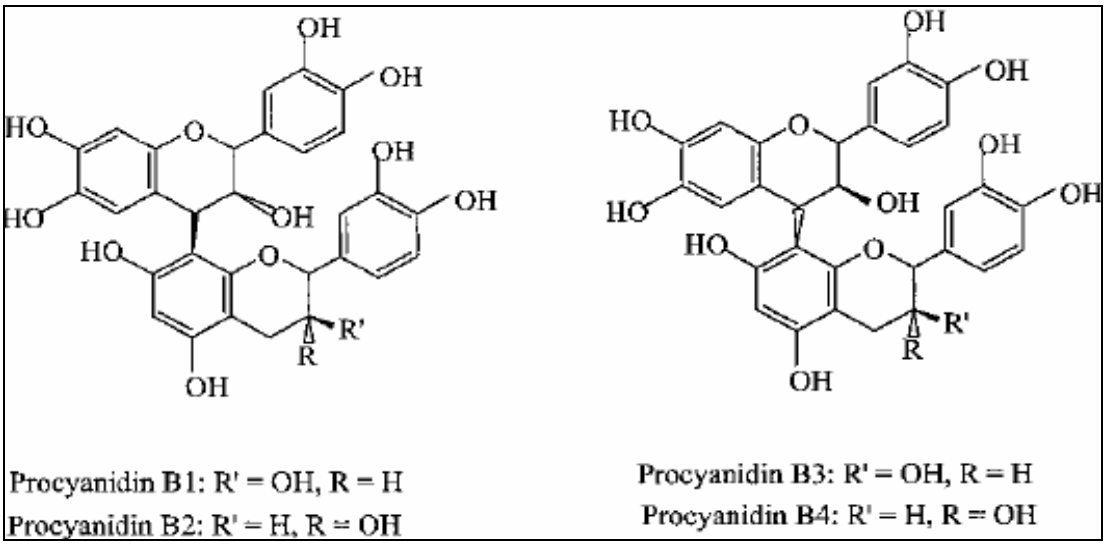

Figure-3: Procyanidin Dimers [Adapted from Shi et al, 2003]

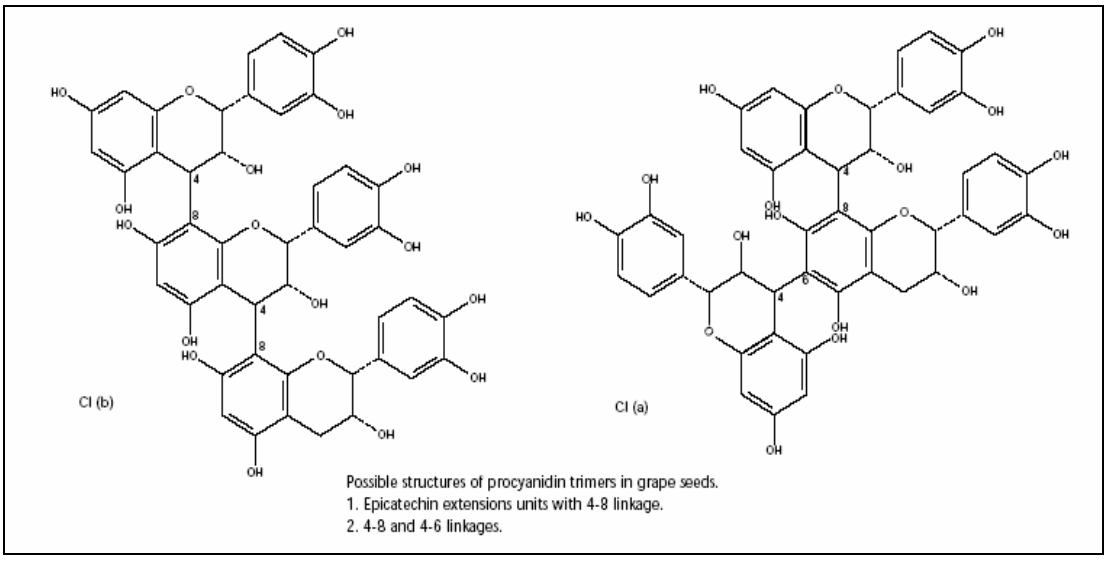

Figure-4: Procyanidin Trimers [Adapted from Vinox Technical Publication-I]

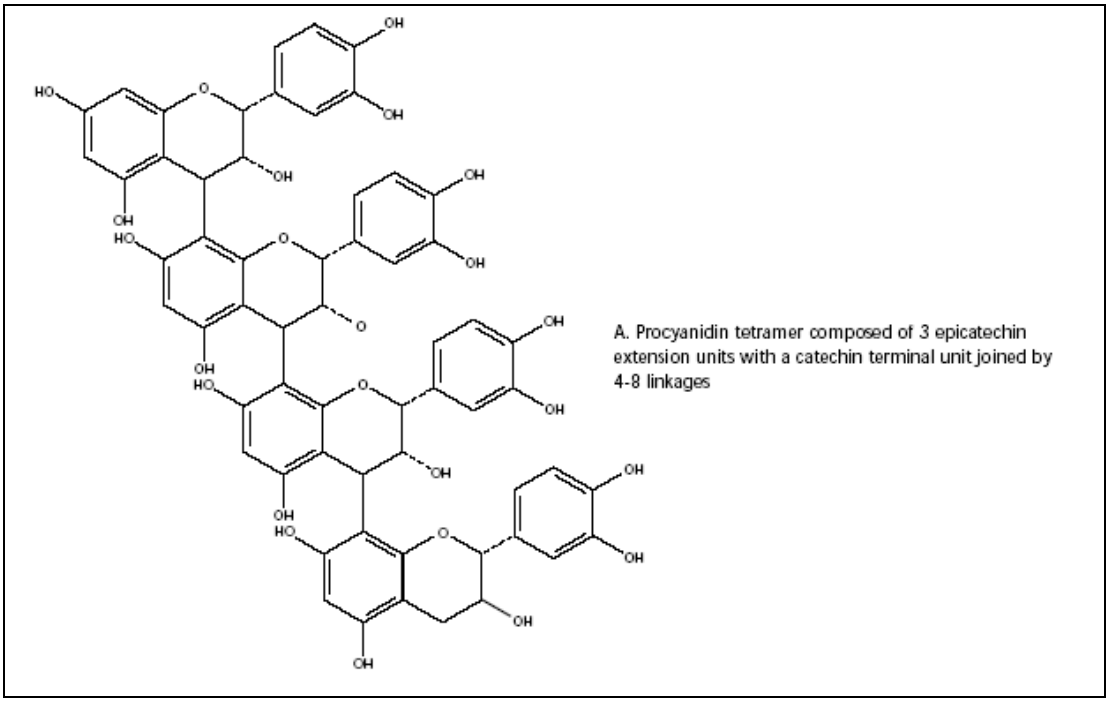

Figure-5: Procyanidin Tetramer [Adapted from Vinox Technical Publication-I]

The molecular weights of three monomers, catechin, epicatechin and epicatechin-(3-O) gallate have molecular weights of 293, 294 and 445, respectively. Depending on degree of polymerization, the molecular weights of procyanidin dimer, trimer and tetramer are 580, 870 and 1,160, respectively.

Structural differences and degree of polymerization affects solubility properties and antioxidant activities of grape phenolics. In general, catechins (monomeric units) are much more soluble in organic phase whereas procyanidins (polymeric units) in aqueous phase [Bonilla, Mayen, Merida and Medina, 1999; Shi et al, 2003]. Polymeric polyphenols are more potent antioxidants than simple monomeric ones [Hagerman et. al., 1998]. Yamaguchi and coworkers (1999) observed that as the degree of polymerization leads to increase superoxide-scavenging ability of phenolics increases. In addition, antilipoperoxidant effect of phenolics depends on the number and position of hydroxyl and methoxyl groups in the benzene ring and on the possibility of electron

delocalization in double bond [Milic et al, 1998]. On the other hand, the phenolic content of a fresh grape variety is affected by four agro-ecological factors [Revilla et al, 1997]:

- Cultivar
- Year of production (climatic conditions)
- Site of production (effect of geographic origin of grapes, soil chemistry and fertilizers used)
- Degree of maturation

All of these factors eventually affect the composition and antioxidant property of grape pomace. The phenolic maturation of a fresh grape variety, which can be critical in phenolic content of extracts, is depicted in Figure-6. The effects of cultivar are indicated in Table-1.

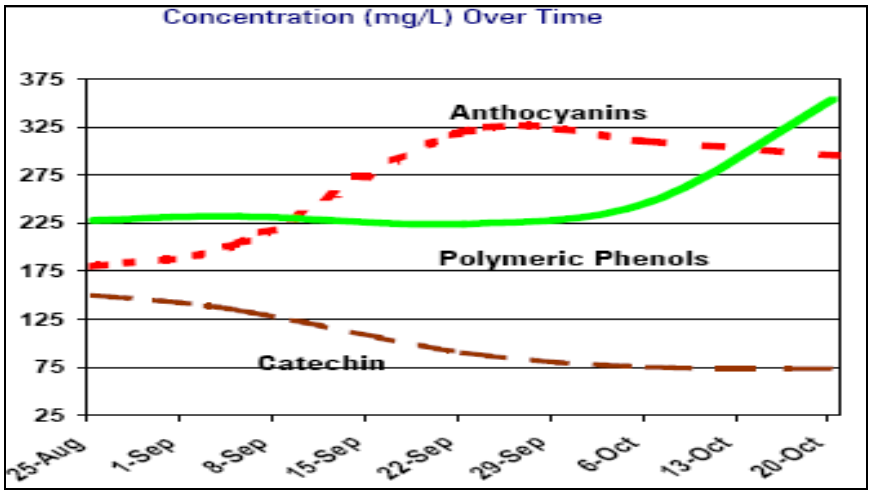

Figure-6: Compositional Change of Grape Ingredients over Time [Adapted from Technical Bulletin of ETS Laboratories]

Table-1: Different Cultivars and Phenolic Contents

		Level (mg /100 g)			
Cultivar	**Color**	**Monomers**	**Dimers**	**Dimer Gallates**	**Trimers**
Cabernet	Red	232	169	43	26
F. Cabernet	Red	125	95	11	32
Sauvignon	Red	228	375	108	67
Merlot	Red	143	97	37	23
Pinot Noir	Red	437	235	41	84
Chardonnay	White	141	126	17	9
DeChaunac	Red	213	40	4	17
Baco Noir	Red	204	292	54	53
M. Foch	Red	88	141	24	10
Vincent	Red	439	238	54	28
Brights 12	Red	75	40	14	9
V 65115	Red	119	30	7	18
Seyval	White	44	16	Tr	Tr
Concord	Red	125	98	13	10
Elvira	White	95	45	7	5
Niagara	White	155	49	10	17

Table-1 was constructed form the data published by Fuleki and Silva [1997]. Monomers expressed as (+)-catechin equivalents; dimers and timers as procyanidin B2 equivalent; and dimer gallates as B2-3'-O-gallatate equivalent. "Tr" denotes the trace amount. As observed, amounts of the constituents can be very different for different grape cultivars.

1.4 RED WINE PRODUCTION and GRAPE POMACE♦

In Figure-7, red wine production process is shown. As it can be seen, fresh grape is first sent to the bundler. In this unit, bunches of grapes are mechanically separated. After separation, grapes are transferred to maceration tanks, yeast is added and the fermentation starts in this unit. If the Merlot type grape is used in the maceration process, typical maceration time varies between 11 and 25 days depending on the desired product. During the maceration, temperature is kept at closely around 25°C. At the end of the maceration time, the liquid-solid mixture is filtered and solid particles are sent to the presses whereas liquid phase (grape juice) is transferred to fermentation tanks. Fermentation is continued in this unit for 10 to 20 days at 14°C to 16°C. The control of acidity of fermentation medium (pH) is adjusted by addition of malic acid. Throughout the operation, temperature control is very important, because formation of side products directly affects the quality of wine. Grape juice obtained from presses and that coming from the fermentation tanks are transferred to collage and stabilization unit. In this unit, wine is clarified to eliminate the precipitates by the aid of clarifying agents. During the entire process, filters are used to eliminate precipitates. At the end of this period, wine is transferred to aging tanks. Aging is very important period and that influence the quality of wine. Each wine type has own aging period. In the aging operation, the volume and construction material of tank used in storage of wine is also very important. Stainless steel barrels do not any desirable effects on the wine, but oak barrels affect the quality of wine. In addition, aging in oak barrels is faster than that in steel barrels. During the aging period, taste, color and smell of wine changes. These changes are controlled by periodic laboratory analyses. Aging operation is realized at 18°C for specific period of time depending on desired quality of product. At the end of aging period, microfiltration is carried in order to remove tiny precipitates and yeast if there exists in the wine.

♦ This section of report was constituted by observations of technical visit to Kavaklıdere Ankara Plant

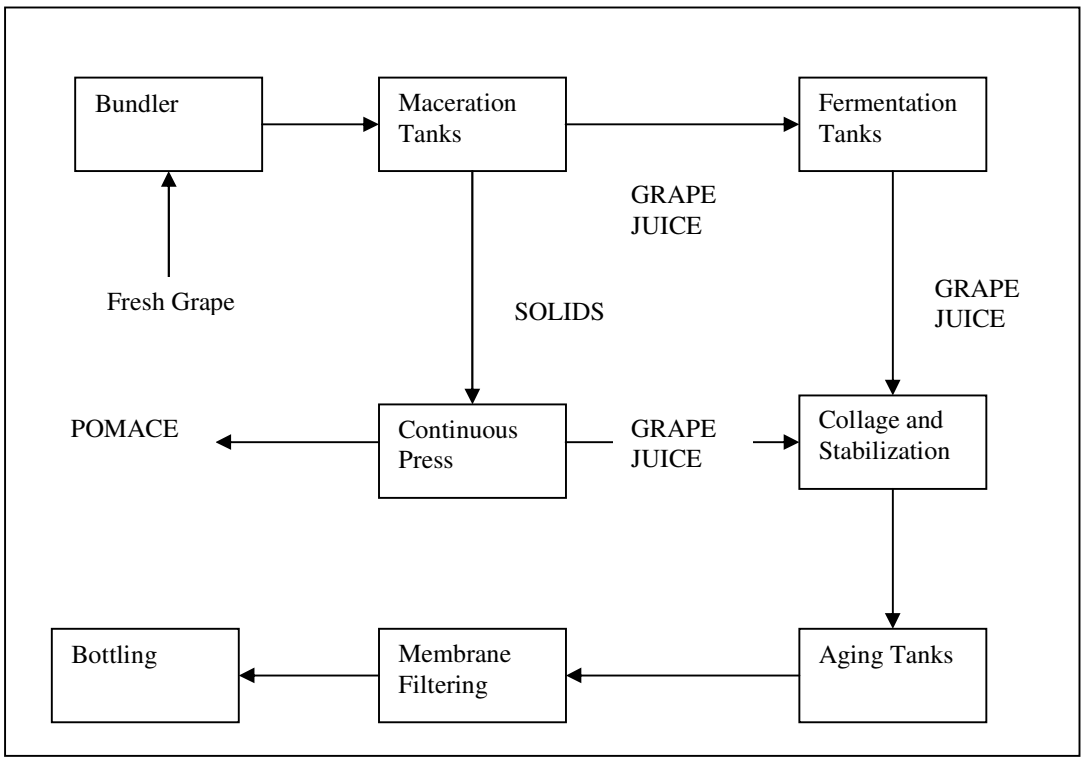

Figure-7: Red Wine Production Process

As it can be seen from Figure-7, grape pomace, which was used as raw material in this study, is press residue of wineries. Since it is coming from maceration tanks to presses, it is partly fermented. The ingredient of pomace is depended on the both raw material used and process conditions. Pomaces obtained from different grape cultivars or the same cultivar but different maceration conditions can affect the amount of phenolics and other constituents in the pomace. For the sake of comparison, Escarpa and Gonzales (2001) reported that red wine pomace might include up to 13 mg/g sugar and 1.8 mg/g protein. In addition, weight percent of volatiles and ash content of pomace was reported as 64.8 % and 8.1 % respectively [Encinar, Beltran, Ramiro and Gonzales, 1998].

On the other hand, the grape production in Turkey between the years 1998 and 2002 is indicated in Table-2. Approximately, 3 % of produced grape, 105,000 tons, has been used in wine production. In addition, approximately 250 kilograms of pomace is discharged per ton of fresh grape used in plant. Therefore, annual pomace discharge is about 26,250 tons.

Table-2: Total Grape Production in Turkey

[Adapted from Government Statistics Institute]

YEARS	**PRODUCTION (1000 tons)**
1998	3,700
1999	3,600
2000	3,600
2001	3,250
2002	3,500

1.5 ULTRASOUND ASSISTED EXTRACTION

Sound waves are mechanical vibrations that need matter to travel. Requirement of matter for traveling is the basic difference of sound waves from electromagnetic waves. Therefore, as sound waves move, they create expansion and compression cycles within the medium. In an expansion cycle, molecules are moved apart whereas in a compression cycle they come together [Luque-Garcia & Luque de Castro, 2003].

The frequency of ultrasound is higher than the audible range to humans. Thus the lowest ultrasonic frequency is accepted as 20 kHz [Luque-Garcia & Luque de Castro, 2003].

“In a liquid, the expansion cycle produces negative pressure. If the ultrasound is strong enough the expansion cycle can create bubbles and cavities in the liquid. This is so when the negative pressure exerted exceeds the local tensile stress of the liquid, which varies depending on the nature and purity. The process by which vapor bubbles from, grow and undergo implosive collapse is known as cavitation. The whole process takes place within about 400 microseconds” [Luque-Garcia & Luque de Castro, 2003].

In fact, cavitation is a nucleated process. In other words, it occurs at pre-existing weak points, such as gas filled crevices in suspended particulate matter, in the liquid and most of the liquids include sufficiently high amounts of small particles for cavitation [Suslick, 1994].

After a certain time, bubbles cannot continue to absorb energy from ultrasound and cannot grow and so collapse. Rapid adiabatic compression of gases and vapors within the bubbles produces extremely high temperatures [Luque-Garcia & Luque de Castro, 2003].

Suslick (1994) estimated that temperature of these hot spots could reach 5000°C and the pressure could reach 1000 atm. However, this extremely high amount of heat produced cannot affect the bulk conditions, because the bubbles are very tiny and the heat is dissipated to the medium in very short period of time. In other words, the rate of cooling after implosion of bubble has been estimated as 10 billion °C per seconds [Luque-Garcia & Luque de Castro, 2003].

Ultrasound assisted extraction is depended on the destructive effects of ultrasonic waves. The possible advantages of ultrasound in extraction are as follows [Vinatoru et al, 1997]:

- Intensification in mass transfer
- Cell disruption
- Enhanced penetration
- Capillary effects

Enhancement in mass transfer arises from creation of very high effective temperatures, which increase the solubility and diffusivity, and pressures that favor penetration and transport [Luque-Garcia & Luque de Castro, 2003].

Ultrasound assisted extraction of various analytes from a variety of organic and inorganic samples using different types of solvents have been found in the literature. Ultrasonic bath and ultrasonic probe systems are the two most common devices used in ultrasound-assisted extraction. Although ultrasonic baths are more widely used devices, they have two main drawbacks that considerably decrease experimental repeatability and reproducibility [Luque-Garcia & Luque de Castro, 2003]:

- Lack of uniformity in distribution of ultrasound energy (only a small fraction of total liquid volume in the immediate vicinity of the ultrasound source experiences cavitation)
- Decline of power with time, so the energy supplied to bath is wasted

In this thesis study, ultrasonic bath was used in order to disturb the cell walls of grape pomace thereby providing enhancement in mass transfer in spite of existing drawbacks of ultrasonic bath.

1.6 PREVIOUS STUDIES

1.6.1 Health Aspects

Grape seed extract (GSE) is one of the approved natural food additives accepted in Japan. It is used in processed fishery foods as an antioxidant at a concentration of 0.01-1.0 % [Nakamura, Tsuji, Tonogai, 2003]. It has also been indicated that phenolics extracted from grape seeds had antibacterial activity [Jayapraksha, Selvi and Sakkariah, 2003; Baydar, Özkan and Sağdıç, 2003], that they were effective especially in gram-positive bacteria cultures [Jayapraksha et al, 2003], and that there was a proportional

relationship between antibacterial effect and amount of total phenolics extracted from grape seeds [Baydar et al, 2003]. On the other hand, Negro, Tomassi and Micelli (2003) studied the preventive effects of ethanolic grape seed extract on oxidation of β-carotene-linoleic acid system. Their results have indicated that grape seed extract has preventive effects on lipid peroxidation comparable with BHA and the preventive effects depend strongly on the amount of total phenolics in the extract.

Phenolics in grape seeds have additional desirable effects on human health. Grape seed procyanidins have been reported to be potent free radical scavengers and were found to exhibit novel cardioprotective properties [Bagchi et al, 2003; Aldini, Carini, Piccoli, Rossoni and Facino, 2003]. In a recent in vivo study, preventive effects of grape seed procyanidin extract (GSPE), vitamin E and C, and β-carotene were compared in lipid peroxidation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and DNA fragmentation in the brain and liver tissues of mice [Bagchi et al, 2000]. They found that GSPE had much stronger protective effects on tissues than vitamin E, vitamin C, combination of vitamin E and C, and β-carotene Furthermore, the work of Frankel, Waterhouse and Tussedre (1995) indicated inhibitory effects of GSE on oxidation of human low-density lipoprotein (LDL) in vitro. In addition, Saito and coworkers (1998) reported that anti-ulcer activity of grape seed extracts including either high or low flavanol contents.

1.6.2 Extraction Conditions

In one of the most recent studies, Caillet, Salmiéri and Lacroix (2006) evaluated the total phenol contents and free radical scavenging abilities of commercial grape seed extracts, namely, MegaNatural^{TM} Whole Grape Extract, Grape Seed Extract and Grape Skin Extract. They have reported that free-radical scavenging activities of extracts decreased as their polarities decreased.

Semi-continuous extraction conditions of phenolics from Garnacha (a white grape variety) pomace were investigated by Pinelo, Fabbro, Manzocco, Nunez and Nicoli

(2005). The effects of process parameters, namely, amount of sample, size of particles and flow rate of solvent, which was water, on amount of total phenolics extracted and antioxidant properties of extracts were analyzed. They reported that maximum amount of phenolics was obtained at low flow rate, small particle size and low amount of sample conditions.

Palma and Taylor (1999) studied on the extraction of phenolic compounds from grape seeds with near critical carbon dioxide. They were investigated the effects of CO_2 density, modifier type, percent modifier and extraction temperature. They also compared the results with aqueous methanol extraction for 16-24 hours and ultrasound assisted extraction with aqueous methanol for one hour. They reported that approximately 16 % higher recoveries than the other two methods had obtained in supercritical fluid extraction. They suggested that possibility of some sort of degradation in ultrasound assisted and conventional liquid-solid extraction.

Jyaprakasha, Singh and Sakkariah (2001) worked on the effects of solvent type on extract yield and antioxidant activity in the extraction of Bangalore blue grapes with conventional extraction methods, namely, soxhlet extraction and solvent extraction. They have reported that use of single solvent, such as acetone or methanol, gave high yield of extract but low antioxidant activity compared to the use of ethyl acetate or ethyl acetate water mixture, whose yield was low but antioxidant activity was higher.

Yılmaz and Toledo (2005) were worked on the effects of different solvents on the amount of total phenolics in extracts and antioxidant activities of extracts by oxygen radical absorbance capacity with different grape varieties. They reported that aqueous solutions of ethanol, methanol and acetone were better solvents than pure ones.

Phenolic content of grape pomace extracts are expected to strongly depend on extraction conditions. Reason for such dependency mainly arises from the position and the form (soluble, suspended, colloidal) of grape phenolics in cellular level. The soluble

phenolics located in vacuoles are preferentially extracted during winemaking process, leaving those combined with cell walls behind [Robards, Antolovich, Prenzler and Ryan, 2000]. The extraction parameters should therefore be adjusted accordingly. In classical extraction methods, the extraction solvent, temperature, contact time, solid-liquid ratio and particle size are the major parameters that affect the quality of the extract and process efficiency.

Several studies about the analytical methods for phenolics emphasize the use of methanol to be best solvent, yet ethanol (EtOH) and water are the most widely applied extraction solvents in food systems because of the hygiene, low cost and abundance in addition to being compatible with health [Moure et al, 2001]. It was reported that the extraction of catechins and procyanidins was more efficient when both the ethanol content of the water-ethanol extractant and operation time increased [Alonso, Bourzeix, Revilla, 1991]. On the other hand, grape pomace contains a variety of phenolics; hence the total amount of phenolics and their composition in the extract strongly depend on the solvent used. Procyanidins have been reported to be soluble in the aqueous phase and catechins in the organic phase [Bonilla et al., 1999; Shi et al., 2003]. Therefore, applying a solvent system with medium polarity instead of using pure solvents seems to be more appropriate to maximize amount of total phenolics in extract.

Temperature affects the operation in two ways. While higher temperatures lead to increase in diffusion coefficient and generally in the solubility of solute, too high temperatures may result in degradation of phenolic substances [Palma & Taylor, 1999]. Constituents and particle size of the raw material are also important considerations in overall process efficiency. As stated before, the distribution of total extractable phenolics of fresh grape was found about 10 % in pulp, 60-70 % in seeds, and 28-35 % in skin [Shi et al, 2003]. Thus, separating and using grape seeds alone as a raw material would give lower results in total phenolics than grape pomace. Reduction in particle size should favor solvent extraction and increase in total phenolics as reported by Bonilla et al. (1999) and Pinelo et al. (2005).

Another parameter important in mass transfer is the liquid-solid ratio. Increase in the ratio favors the mass transfer driving force, but relationship between extract yield and liquid-solid ratio is not completely linear. In a recent research study, effects of liquid to solid ratio in extraction of polyphenolics from tea leaves have been analyzed and the results indicated that 20:1 liquid-solid ratio was sufficient for high extraction [Pan, Niu and Liu, 2003].

One of the most important disadvantages of classical extraction methods (e.g. soxhlet extraction for analytical purposes and solvent extraction for analytical or commercial purposes) is the requirement of several hours of contact times. In a recent study [Albu, Joyce, Paniwynk, Loirmer and Mason, 2004], application of ultrasound in extraction of antioxidants from rosemary has been studied. Operation efficiencies of ultrasonic bath extraction, ultrasonic probe system and shaking water bath system have been compared at different temperatures and different solvents. In all cases, application of ultrasonic bath and probe systems dramatically decreased the operation time. Similar behavior was reported by Cho and coworkers (2005) in extraction of resveratrol from grapes. Independently, Luque-Garcia & Luque de Castro (2003) had already suggested that the application of ultrasound has favorable effects in extraction processes due to creation of high effective temperatures and pressures at the interface between solution subjected to ultrasonic energy and solid matrix. However, prolonged application of ultrasound may result in denaturation of phenolics.

Analysis and characterization of antioxidants or phenolics in extract is a crucial step in all studies. A variety of analysis methods are available in literature. There is not, however, any single accepted method that completely describes antioxidant functionality and efficacy in food systems or living systems. A potent antioxidant in one system may fail to protect or even may cause the damage in another system [Aruoma, 2003], and there may be conflicting results between antioxidant activity, reducing capacity and free radical scavenging ability. Becker, Nissen and Skibsted (2004) have explained these differences depending on several factors such as the physical structure

of the test system, the nature of substrate for oxidation, the presence of interacting components, the mode of initiating oxidation and the analytical method for measuring oxidation. Furthermore, from thermodynamic point of view, free radical scavenging activity and antioxidant property are not necessarily closely connected [Frankel, 2005]. Free radical scavenging ability depends on the presence of unpaired electrons, whereas antioxidant property is determined by the oxidation-reduction potential [Havsteen, 2002]. Recent studies indicate the strong correlation between amount of total phenolics and Ferric Reducing Antioxidant Power (FRAP) [Benzie & Szeto 1999; Katalinic& Milos, 2006]. Furthermore, Yılmaz & Toledo (2005) have reported strong correlation between the amount of total phenolics and oxygen radical absorbance capacity. On the other hand, complex systems and methods of analysis to cover all such aspects may not be realistic [Frankel & Meyer, 2000]. There should be a close link between the purpose of use of the purified compound and the test system. However, there is no strong agreement between the ultimate compound, purpose of its use or environment (in vitro or in vivo system), and test system or analysis method. It is nevertheless practical and informative to indicate the effects of process parameters in extraction even if there is serious criticism on analyzing total phenolics because of inadequacy in characterization of extract.

1.7. RESPONSE SURFACE METHODOLOGY

1.7.1 General Considerations

Most of the experiments either test a hypothesis or study on the response characteristics of a system. Hypothesis testing experiments are generally asking whether there is a true difference between two or more items. However, response surface experiments attempt to characterize the output or response of a system as a function of explanatory variables [Thompson, 1982].

Response surface methodology (RSM) consists of a set of statistical methods that can be used to develop, improve, or optimize the processing conditions [Myers & Montgomery, 1995]. RSM typically is used in situations where several factors influence one or more performance characteristics, or responses of the system. In addition, RSM may be used to optimize one or more responses.

The response can be assessed as a surface over the explanatory variables' experimental space. Therefore, the term response surface has been associated with experiments planned to identify or assess one or more response variables as a function of the independent variables [Thompson, 1982]. In order to relate the response with explanatory variables and create a surface, a function related to these variables is needed. Today, many scientists have argued that when an appropriate model based on reasonable assumptions has been developed, it should be used instead of a general response model. If there is no such a model equation, polynomials of first degree and second degree are the most frequently used response functions [Thompson, 1982].

First order models are generally used for screening experiments. The purpose of screening experiments is to identify the most significant explanatory variables that affect the response. In most cases, second order or quadratic functions are sufficient to characterize the effects of explanatory variables on response. Second order models can be formed by the addition of the terms to first order models. In this type of model, estimates of parameter values in the polynomial equations can be made by the method of least squares without complex calculations. On the other hand, polynomial models have following disadvantages [Thompson, 1982]:

- Extrapolation outside the range of independent variables is impossible.
- The second-degree polynomial is symmetrical about the optimum. However, the true response of the system may not be symmetric.

- The second-order model does not include a form that can asymptotically approach a constant response level. This response form is frequently encountered in biological and agricultural data.

In spite of possible disadvantages, if there is no mechanism based model available or the available model requires more parameters or if it is nonlinear in the parameters, a more empirical (second order model) may be favored.

The order of experimental units (run or points) and the explanatory variable levels for each run are specified by experimental designs. By using coded level for each variable, the designs are dependent only on the number of variables and selected response equation [Thompson, 1982].

In response surface experimentation, it is desirable to estimate a pure or experimental error term and lack-of-fit error term. The variation experienced when repeating a run at the same design point determines the pure error. The level of variation may change in different locations of the experimental space or it may change with time. If it is known to be pure error is independent of time, it can be estimated before or after the experiment. If it is considered to be reasonably independent of location in the experimental design space, it can be estimated from replications of single point. In general, pure or experimental error term is estimated from replicated experiments at center point (all independent variables with a coded level as zero). This assumes that the pure error term may change with time but it will be reasonably uniform throughout the experimental region [Thompson, 1982].

1.7.2 Second Order Central Composite Design

Central composite designs include three types of experimental points [Thompson, 1982]:

1.7.2.1 Factorial Points

If number of explanatory variables or independent variables is represented by k, the number of factorial points will be

$$n_c = 2^k \qquad (1)$$

These points are located at the vertices of a square, cube, hypercube or a fraction of hypercube. The coded independent variable levels of these points are ± 1.

1.7.2.2 Star Points

These experimental points have coordinates of (± α, 0,…,0). The number of these points (n_a) is

$$n_a = 2k \qquad (2)$$

Frequently the value of α is selected to make the design rotatable. A rotatable design has uniform variance at any given radius from the center of design. The rotatable condition is satisfied by

$$\alpha = (n_c)^{1/4} \qquad (3)$$

1.7.2.3 Replicated Points

Experimental points belonging to this group represent the replication at center point conditions. These points all have the coordinates (0,…,0). These points provide a means for estimation of the experimental error and provide a measure of lack-of-fit. The number of this group of points are found by

$$n_o = \frac{4\alpha^2 (n_c + \alpha^2)}{n_c} - n_a \qquad (4)$$

The number of points specified by this equation should be rounded to the nearest integer number.

In this thesis study, 2nd Order Central Composite Design was used. The details of the design were stated in Experimental Design part.

In the light of these considerations, the aim of this study was the determination of the best ratio between water and ethanol as the solvent along with the relationship between durations of ultrasonication and shaking water bath treatments in a sequential manner in order to maximize total phenolic content of grape pomace extracts. For this purpose, the effects of sonication time, shaking water bath time and the composition of the aqueous ethanol have been studied by response surface methodology.

CHAPTER 2

MATERIALS and METHODS

2.1 MATERIALS

Pomace is the press residue of wineries. Merlot type red grape pomace, obtained from Kavaklıdere Şarapları A.Ş., was used as raw material in this study. The raw material had been macerated for 15 days at 25°C before pressing, and it was received as wet.

For extraction, absolute grade ethanol (Delta) and ultra pure water (Milli Q, Millipore) was used, and for liquid-solid separation purposes, trichloroacetic acid (Beaker) was purchased. Gallic acid (Sigma), anhydrous sodium carbonate (Merck), and Folin & Ciocalteu's Phenol Reagent (Sigma) were used in analyses.

2.2 SAMPLE PREPARATION and PRETREATMENT

2.2.1 Drying and Moisture Content Determination

The pomace was dried at 50°C for 24 hours in a tray dryer with air circulation. The moisture content of dried pomace was determined by drying at 105°C for 2 hours. The detailed procedure used in moisture content determination is given as Appendix A1.1. The pomace consisted of approximately 65 % seeds and 35 % skins and very small amounts of stems, as determined by simple mechanical separation. Dried pomace was stored under ambient conditions.

2.2.2 Size Reduction and Sieve Analysis

The raw material was ground in a cutting mill to size less than 850 microns (20 mesh) and size distribution was determined. Preliminary studies indicated that storing ground grape pomace in a tightly closed non-transparent glass bottle in a refrigerator at + 4°C prevents air and light effects up to 3 days, yielding no significant loss in phenolics. The procedure used in size reduction and sieve analysis is given in Appendix A1.2.

2.2.3 Ash Content

Ash refers to inorganic residue remaining after either ignition or complete oxidation of organic matter in a foodstuff. Dry ashing technique, which refers to vaporizing water and other volatiles and burning organic substances in the presence of oxygen in air to CO_2 and oxides of N_2, was employed. The ash content of the pomace was determined by keeping a sample at 600°C for 4 hours [Harbers, 2003]. The details of the procedure are given in Appendix A1.3.

2.3 EXTRACTION of PHENOLICS

Desired compounds from food matrices are usually extracted by conventional techniques such as solvent extraction, while the application of ultrasound is generally suggested for some analytical determinations [Luque-Garcia & Luque de Castro, 2003]. In this study both techniques are utilized in the extraction of total phenolics from grape pomace. An ultrasonic bath (Branson 2200, 47 kHz working frequency) and a shaking water bath (Nuve ST 420) were used in a sequential manner. The temperature of the shaking water bath was chosen as 45°C to prevent denaturation of phenolics. Pure water, pure ethanol or their mixtures were the extraction solvents. In each run, 5 g of powdered grape pomace were added in a conical flask containing 100 mL of solvent.

The flask, equipped with a thermometer, was kept first in the ultrasonic bath and then in the shaking water bath (Appendix A1.4 and A1.5).

The effects of sonication time in ultrasonic bath (ST), shaking water bath time (WBT) and volume percentage of EtOH in solvent (SC) on amount of total phenolics were investigated in this study. Sonication in ultrasonic bath was the first step of extraction. The aim was to develop an extraction procedure with a reasonable contact time so that retention times in water bath were selected to be less than 40 min. The grape pomace contains a variety of phenolics, probably with different properties and polarities, and thus the solvent composition was varied over the whole range of 0 % to 100 % ethanol.

2.4 EXPERIMENTAL DESIGN

Three process variables, namely sonication time (X_1), water bath time (X_2) and composition of solvent as volume percentage of EtOH (X_3), were studied on five levels to investigate their effects on amount of total phenolics in the extract by 2^{nd} order orthogonal and rotatable central composite design with 8 cube points, 6 star points and 9 replications of the center as described before.

As shown in Figure-8, the lowest and the highest values of each parameter is coded as – 1.682 ($-\alpha$) and + 1.682 ($+\alpha$), respectively. Specification of the lowest and the highest values determines the remaining part of the design.

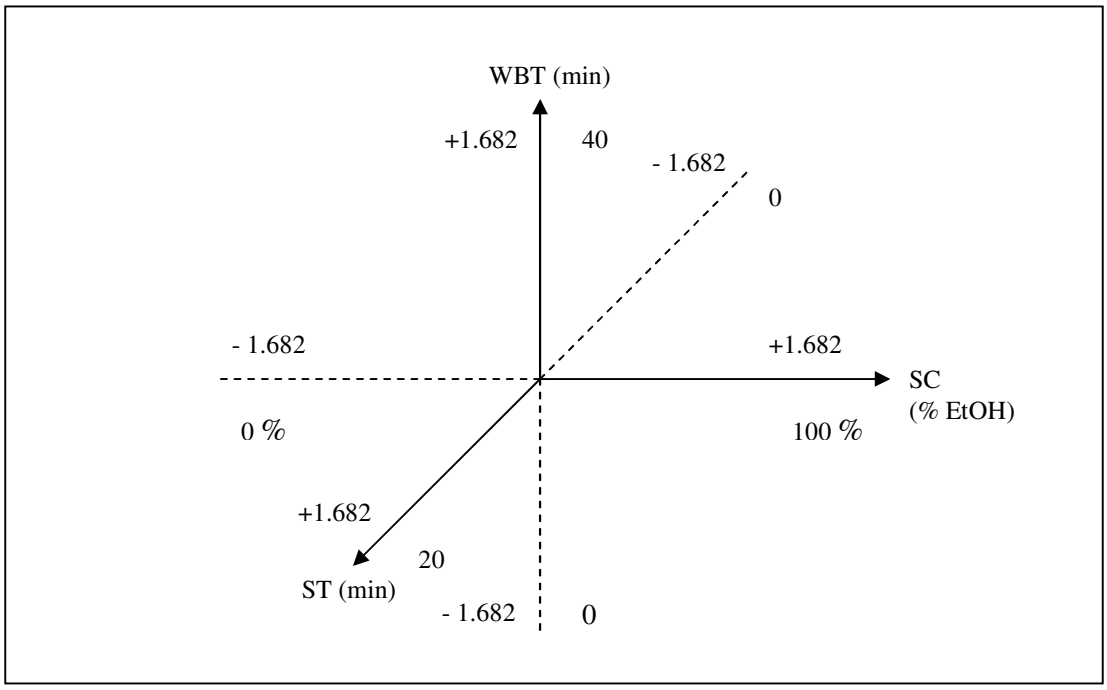

Figure-8: Codification of Experimental Parameters

The mid value of each parameter coded as 0; parameter levels corresponding –1 and +1 are found by linear proportion.

Table-3 indicates the design matrix with variables in both coded and non-coded form for easier comparison.

Table-3: Experimental Design Matrix

RUN	Parameter Level			Coded Level		
	ST (X_1) min)	**WBT (X_2) (min)**	**SC v/v (X_3) (% EtOH)**	**x_1**	**x_2**	**x_3**
1	14	28	70	1	1	1
2	14	12	70	1	-1	1
3	14	28	30	1	1	-1
4	14	12	30	1	-1	-1
5	6	28	70	-1	1	1
6	6	12	70	-1	-1	1
7	6	28	30	-1	1	-1
8	6	12	30	-1	-1	-1
9	0	20	50	-1.682	0	0
10	20	20	50	+1.682	0	0
11	10	0	50	0	-1.682	0
12	10	40	50	0	+1.682	0
13	10	20	0	0	0	-1.682
14	10	20	100	0	0	+1.682
15	10	20	50	0	0	0
16	10	20	50	0	0	0
17	10	20	50	0	0	0
18	10	20	50	0	0	0
19	10	20	50	0	0	0
20	10	20	50	0	0	0
21	10	20	50	0	0	0
22	10	20	50	0	0	0
23	10	20	50	0	0	0

In Table-3, Run-1, for example, indicates 14 minutes of sonication in ultrasonic bath, then 28 minutes of shaking in water bath at 45°C when extraction solvent consists of 30 % EtOH by volume. Runs between 15 and 23 are nine replications at center point conditions. The following second order model was used to adjust data on the response surface R.

$$R = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (5)$$

Where β_0 is the value of the objective function under central point conditions, β_1, β_2, β_3 indicate the principal effects of each variable; β_{11}, β_{22}, β_{33} represent the quadratic effects of variables, and β_{12}, β_{13}, β_{23} indicate the cross effects of variables.

2.5 LIQUID-SOLID SEPARATION

In literature centrifugation is accepted as the most accurate way of separating liquid and solid phases.

Immediately after the extraction samples taken from the flask were placed in centrifuge. The samples were then centrifuged at 1750g for 15 minutes in order to separate liquid and solid phases and clarify the extracts.

One of the possible interferences may arise from proteins in the extract. Escarpa and Gonzales (2001) showed that red wine pomace could contain up to 1.8 mg protein / g pomace.

Folin's Reagent can react with both phenols and proteins. In order to prevent possible protein inference in the analysis and to precipitate the proteins, 700 μL of 10 % TCA solution was added to each tube before the centrifugation. The details of centrifugation procedure are given in Appendix A1.6.

2.6 ANALYSIS of TOTAL PHENOLICS in the EXTRACT

The Folin-Ciocalteu method was used to assay total phenolics in this study. The method was originally developed for analysis of amino acid residues, tyrosine and tryptophane [Folin & Ciocalteu, 1927], which was then improved Singleton & Rossi (1965) and Singleton & Slinkard (1977). This method has been cited as being the Association of Official and Analytical Chemists' method for determining the total phenol content in wines [♦]. The Folin-Ciocalteu Reagent is an oxidizing agent consisting of heteropolyphosphotungstate-molybdate. It oxidizes the phenolates, reducing the heteropoly acids to a blue Mo-W. The blue colored product is a mixture of 1-, 2-, 4-, and 6- electron reduction products in tungstate series $(P_2W_{18}O_{62})^{-7}$ to $(H_4P_2W_{18}O_{62})^{-8}$ and 2-, 4-, and 6-electron reduction products in the molybdate series $(H_2P_2Mo_{18}O_{62})^{-6}$ to $(H_6P_2Mo_{18}O_{62})^{-6}$ [♦]. The method described by Singletton & Rossi (1965) and Singletton & Slinkard (1977) was used in determination of the total phenolic contents of extracts. A sample of 20 μL was mixed with 6.5 ml of deionised water. Non-diluted Folin's Reagent (Sigma) of 0.5 ml and then 3 ml of 10 % anhydrous sodium carbonate solution were then added to the mixture, and the final mixture was kept in shaking water bath at 40°C for 30 minutes for color development. The absorbance was measured at 765 nm by Hitachi-3200 UV-Visible Spectrophotomer. Results were reported as gram gallic acid equivalents per liter of solvent (g GAE/L) and milligram of GAE per gram of pomace (mg GAE/g Pomace). The details of the procedure are given in Appendix A 1.7.

2.7 SUMMARY OF THE EXPERIMENTAL PROCEDURE

Figure-9 summarizes the experiments conducted. 5 g of powdered grape pomace and 100 mL of solvent was added into a conical flask, equipped with a thermometer. The flask was first kept in ultrasonic bath. Immediately after the sonication, the flask was kept in a boiling water a few seconds in order to increase the temperature to 45°C,

♦ http://www.activin.com/Testing%20White%20paper__.html

which was the temperature of shaking water bath. Afterwards, the flask was placed in the shaking water bath to continue extraction. At the end of extraction period, two samples were taken from the flask and TCA solution (%10) was added. Then the tubes placed into the centrifuge. Immediately after the centrifugation, analysis of total phenolics was realized colorimetric analyses at 765 nm.

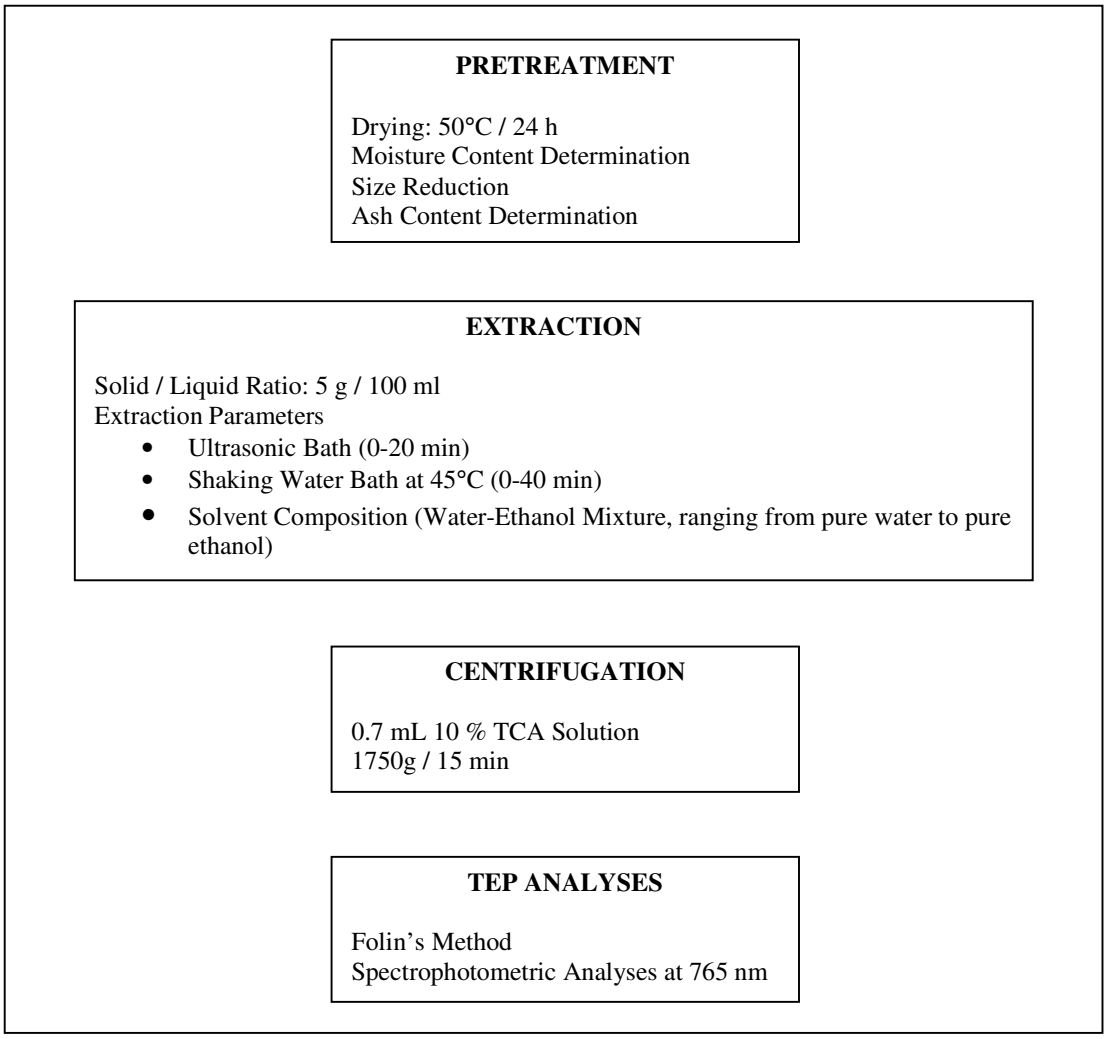

Figure-9: Experiment Flowsheet

CHAPTER 3

RESULTS and DISCUSSION

The moisture content and the ash content of the dried pomace were found to be 4.85 % and 7.1 %, respectively. The results of sieve analysis were indicated in Table-4.

Table-4: Sieve Analysis of Dried Ground Pomace

Size Range (µm)	Amount Retained (%)
- 850 / + 600	12
- 600 / + 250	53
- 250 / + 212	31
- 212 / + 180	3
Pan	1

During the experiments, it was observed that the temperatures of extraction medium varied in the range of 33°C to 40°C depending on the solvent composition and the duration of the ultrasonication. The values for the each run given in the appendix A 2.

Table-5 summarizes the experimental results obtained both in the units of g GAE/L and mg GAE/g pomace. The center point was replicated 9 times for reproducibility and the estimation of pure error in experiments. The percent relative error was found to be 7.70 %, which is reasonable considering the generally poor reproducibilities in ultrasonic baths [Luque-Garcia & Luque de Castro, 2003].

Table-5: Experimental Results

RUN	ST (min)	WBT (min)	SC v/v (% EtOH)	GAE (g/L)	GAE (mg / g pomace)
1	14	28	70	2.10	42.0
2	14	12	70	1.88	38.0
3	14	28	30	1.88	38.0
4	14	12	30	1.80	36.0
5	6	28	70	1.48	29.7
6	6	12	70	1.30	25.5
7	6	28	30	1.40	28.0
8	6	12	30	2.36	47.2
9	0	20	50	1.53	31.0
10	20	20	50	2.30	45.4
11	10	0	50	0.90	18.0
12	10	40	50	1.86	37.2
13	10	20	0	0.32	6.44
14	10	20	100	0.84	17.0
15[(a)]	10	20	50	1.70 ± 0.13	34±2.64

[(a)] Average value 9 replications at center point conditions

The two lowest recoveries of phenolics (6.4 mg GAE/g pomace and 17.0 mg GAE/g pomace) obtained in experimental points 13 and 14 are for the cases of pure water and pure ethanol as solvent, respectively. These results clearly indicate that the use of pure solvents in extraction of phenolics from grape pomace was inefficient. Instead, their mixtures were much more effective as extracting solvent. Similar conclusions about the effects of extracting solvent on recovery of phenolics were drawn by Yılmaz and Toledo (2005). On the other hand, the third lowest recovery (18 mg GAE/g pomace) belongs to point 11, where ultrasonication was applied without a subsequent water bath

treatment. The result shows clearly that ultrasonic treatment alone was not sufficient in the extraction of phenolics, probably because a longer contact time was required for diffusion of phenolics from solid matrix to liquid phase. Ultrasonication seems to be more effective if it is applied together with the shaking water bath in a sequential manner. The recovery of phenolics in the remaining experiments ranged between 25.5 and 47.2 mg GAE /g pomace.

Robards et al. (2000) in his critical review reported the total phenolics in grape extract to be varying in the range of 2 to 20 mg GAE/ g dried pomace. It was also stated in the same review that the upper limit for total phenolics could reach 60 mg GAE/ g dry matter in aqueous methanol extraction. Results found in the present study were close to this upper limit although the starting material is not grape but pomace, which had been in maceration for several days. A probable explanation for this situation may be the increase in operation efficiency resulting from application of ultrasound. Albu et al. (2004) have reported similar trends in ultrasonic treatments. In the classical extraction methods, process efficiency is probably more dependent on the type of solvent used. In addition, Gonzales and Escarpa (2001) reported the phenolics content of red wine pomace to be approximately 8 mg GAE/g. All the results in the present study, except the experimental point 13, are higher than the results given by these researchers.

Yılmaz and Toledo (2005) have studied the effects of solvent composition on total phenolic contents and oxygen radical absorbance capacity (ORAC) of extracts obtained from Merlot, Chardonnay and Muscadine seeds. Extracts were obtained by 15 minutes of sonication and followed by 30 minutes of shaking in water bath at room temperature. They have reported the total phenolics content in Merlot pomace as 2.30 mg GAE /g seed and as 2.35 mg GAE/ g skin with pure water as solvent. If one assumes the pomace to consist of 65% seeds and 35% skin by weight, as in this study, then the reported values are equivalent to 2.32 mg GAE/g pomace. In the present study, 10 minutes of sonication and subsequent 20 minutes of shaking in water bath with pure water as solvent gave a higher result (6.40 mg GAE/g pomace). The difference between the

results may be explained, in addition to different extraction temperatures, by considering the total operation times. As discussed later in more detail, the shorter operation times may be enough for higher recoveries when EtOH content of the extracting solvent is low. They have further reported that total phenolic content in Merlot pomace was about 38.5 mg GAE/g seed and 14.9 mg GAE/g skin with water and acetone mixtures, giving a weighted average of 30.2 mg GAE/g pomace. In the present study, 10 out of 15 sets of experimental conditions yielded higher recoveries.

Baydar et al. (2003) reported that 8 hours of soxhlet extraction with 95% EtOH in solvent gave approximately 30 mg GAE/g pomace of Narince, a local white grape variety in Turkey. White grapes contain smaller amounts polyphenolics, but for the sake of comparison, the closest conditions in the present study are the experimental point 14. The present study gives 17.0 mg GAE /g pomace, obtained with 100% EtOH at about 30°C lower temperature and in sixteen-fold shorter operation time.

Negro, Tomassi and Micelli (2003) worked on industrial red grape pomace obtained from the Negro amaro variety. The drying and size reduction operations they applied to the raw material were similar to those in the present study. Phenolics were extracted by a solvent including 80 % EtOH by volume. They reported that pomace had contained 41.9 mg GAE per gram of dried material. For the sake of comparison, the results obtained from experimental runs 1 and 2, at which solvent includes 70 % EtOH, are well agreed (42 mg GAE / g pomace and 38 mg GAE / g pomace) with the reported result.

The coefficients of the following second order model were evaluated by nonlinear regression option of Statgraphics©. The model was developed for recovery, that is, mg phenolics extracted from per gram of dried pomace.

$$R = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (5)$$

$$R = 80.0 - 2.61X_1 - 2.42X_2 - 0.33X_3 + 0.04X_1^2 + 0.016X_2^2 - 0.005X_3^2 + 0.052X_1X_2 + 0.03X_1X_3 + 0.022X_2X_3 \quad (6)$$

The statistical data of model parameters was obtained from Statgraphics© with the estimations of model parameters. Table-6 and Table-7 summarize the output of Statgraphics©.

Table-6: Model Parameters and P-Values

PARAMETER	ESTIMATE	P-VALUE
β_0	80.00	0.0023
β_1	-2.610	0.0582
β_2	-2.420	0.0245
β_3	-0.33	0.4044
β_{11}	0.040	0.1297
β_{22}	0.016	0.1703
β_{33}	-0.005	0.0165
β_{12}	0.052	0.2055
β_{13}	0.030	0.0875
β_{23}	0.022	0.0288

The p-value of each parameter represents the statistical significance of the parameter. As p-value approaches to zero, the significance of the parameter increases. In most cases p-value smaller than 0.05 or 0.07 is accepted as statistically significant.

Table-7: Analysis of Variance (ANOVA)

Source	**Sum of Squares**	**Degree of Freedom**	**Mean Square**
Model	10342.2	9	1149.14
Residual	1536.48	13	118.191
Total	11878.7	22	

F_{Mod} represents whether a significant relationship exists between the dependent variable and set of all the independent variables. It is defined as the ratio of model sum of squares to the residual sum of squares.

$$F_{Mod} = \frac{10342.2}{1536.48} = 9.72$$

For the present degree of freedom values, corresponding F value is 2.71 [Scheaffer & McClave, 1995], which is smaller than F_{Mod}. Therefore, a significant relationship exists between response and independent variables. In addition, Statgraphics© output indicated the p-value of model as 0.0002, and regression coefficient as 87 %, which represented statistical significance of model.

Before attempting to explain the influences of parameters, it should be noted that extraction conditions not only affect the mass transfer conditions but may also cause reactions in extraction environment. Use of ultrasound is known to generate free radicals and may initiate polymerization reactions in short times [Luque-Garcia & Luque de Castro, 2003] in addition to rupturing cell walls. Denaturation reactions of phenolics are among other possibilities. Polymerization reactions may result in increased apparent phenolic content of the extracts. Pinelo et al (2005) also suggested

that extraction conditions could favor the formation of polymeric phenolics. By this perspective, chemical changes may be accompanying mass transfer.

In Figure-10, Figure-11 and Figure-12, response surface plots are indicated.

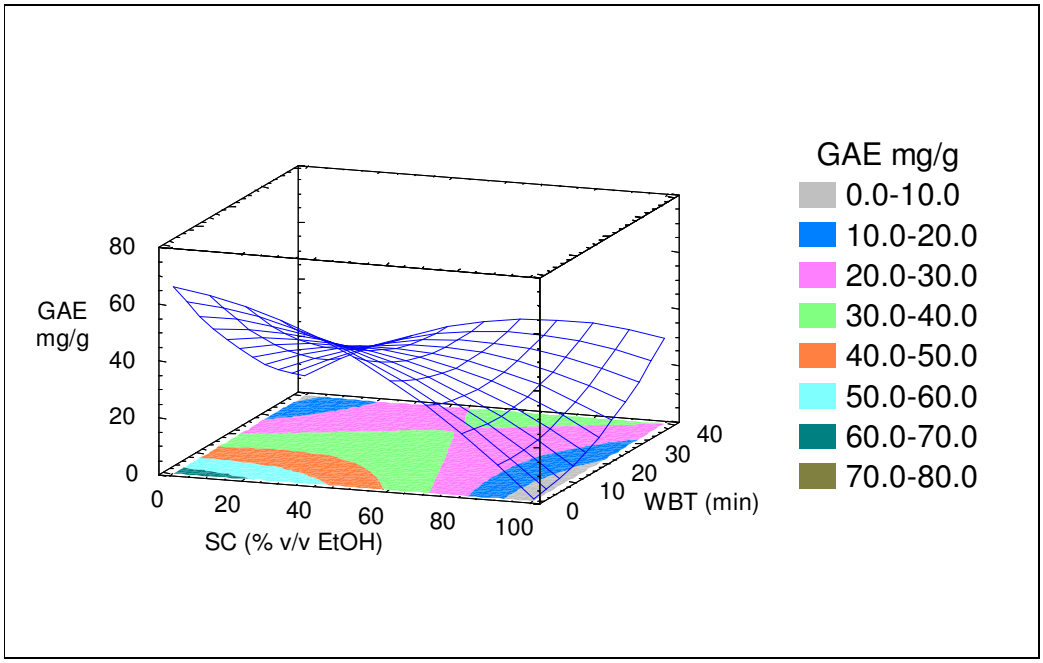

Figure-10: Effects of SC and WBT on Recovery (ST = 6 min)

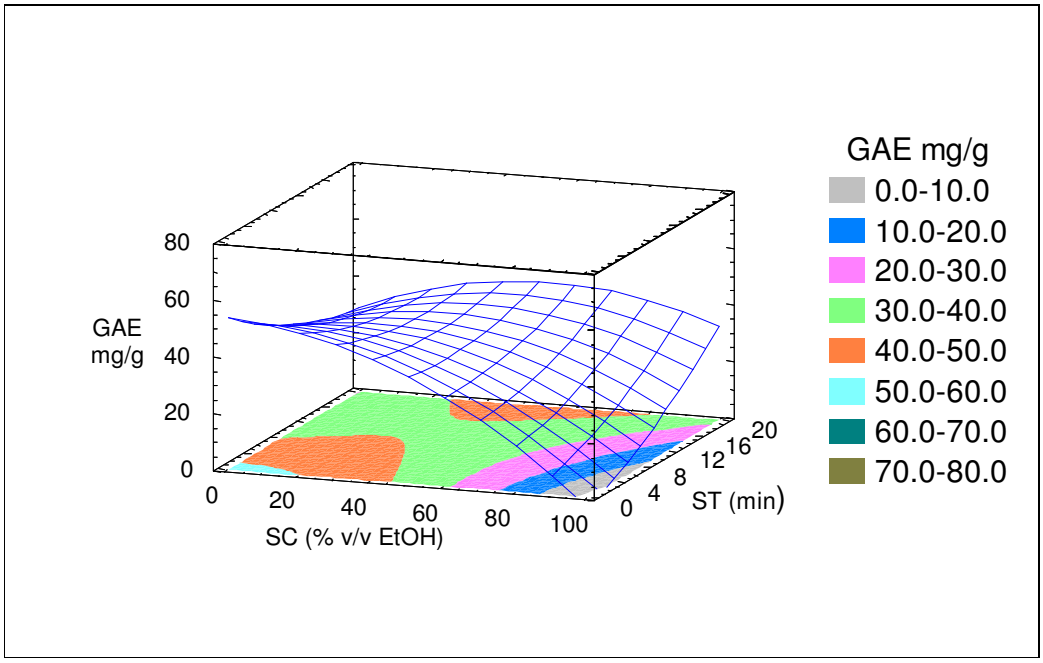

Figure-11: Effects of SC and ST on Recovery (WBT = 12 min)

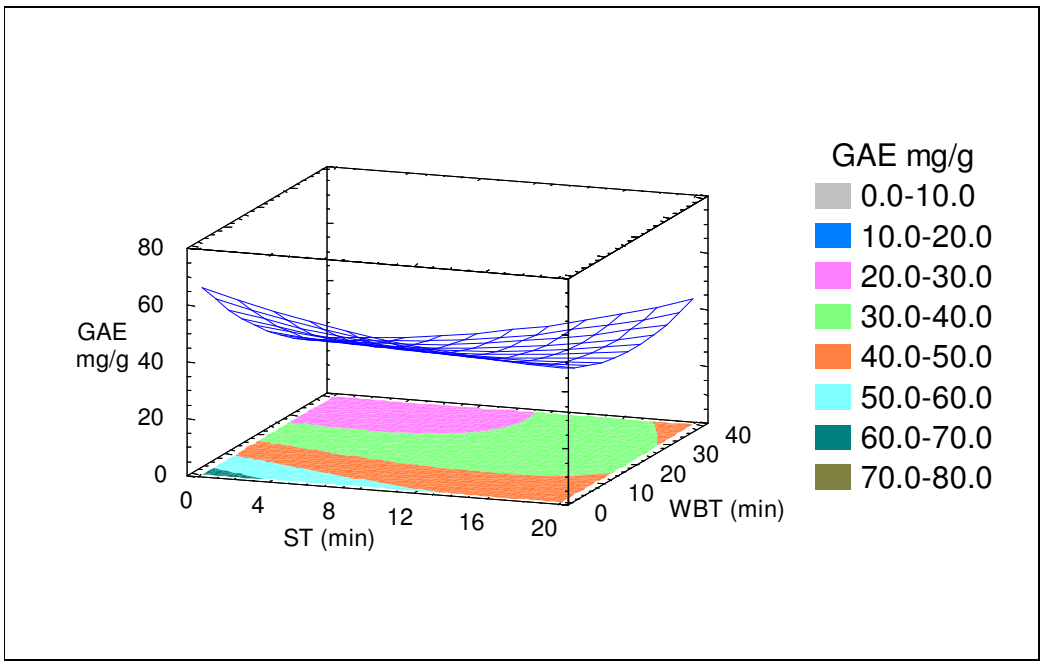

Figure-12: Effects of ST and WBT on Recovery (SC = 30 % EtOH)

As has been stated, polymeric phenolics are more soluble in the aqueous phase, and therefore low EtOH content of solvent is expected to favor higher recoveries of polymeric phenolics. In Figure-10, where ST was 6 minutes, shorter WBT at low EtOH content of solvent and longer WBT at high EtOH content are indicated. The latter is the expected behavior from the mass transfer point of view. Some sort of degradation, which possibly involves water, may explain the negative effect of longer times. Cho et al. [2005] has stated the same argument for ultrasound-assisted extraction of resveratrol from grape stems. The effects of SC and ST on recovery under fixed WBT may be explained with a similar argument. As it can be seen from Figure-11, again shorter ST at low SC, and longer ST at high SC are indicated. A possible explanation to this is again a sort of degradation enhanced by ultrasonication that involves water. The effects of ST and WBT at 30 % EtOH (Figure-12) seem relatively insignificant.

CHAPTER 4

CONCLUSIONS

The present study reveals quite good recoveries of total phenolics from grape pomace by a combination of relatively short times of ultrasonication and shaking water bath treatments. The highest phenolic content in the extracts (47.2 mg GAE/g powder) was obtained using 30 % aqueous EtOH as solvent, applying 6 minutes of sonication and followed by 12 minutes in a shaking water bath at 45 °C. The mathematical model fitted to the data and the response surface plots indicated that even lower EtOH contents of the solvent with shorter sonication and water bath times could give higher results. It is clearly evident that a proper combination of a modern (ultrasound) treatment along with a conventional (shaking water bath) treatment yields a desirable degree of efficiency in extraction operation by increasing mass transfer and possibly enhancing the formation of polymeric phenolics.

REFERENCES

Albu, S., Joyce, E., Paniwnyk, L., Loirmer, J.P., Mason, T.J., (2004). Potential use of ultrasound in the extraction of antioxidants from Rosmarinus officinals for the food and pharmaceutical industry. *Ultrasonics Sonochemistry (11),* 261-265.

Aldini, G., Carini, M, Piccoli, A., Rossoni, G., Facino, R.M., (2003). Procyanidins from grape seeds protect endothelial cells from peroxynitirite damage and enhance endothelium-dependent relaxation in human artery: new evidences for cardio-protection. *Life Sciences (73),* 2883-2898.

Alonso, E., Bourzeix, M., Revilla, E., (1991). Suitability of water-ethanol mixtures for the Vitis vinifera seeds contained in a winery by-product. *Seed Science Technology, 19,* 545-552.

Arouma, O.I., (2003). Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutation Research*, *9* (20), 523-524.

Bagchi, K. & Puri, S., (1998). Free radicals and antioxidants in health and disease, *Eastern Mediterranean Health Journal, 4(2)*, 350-360

Bagchi, D., Bagchi, M., Stohs, S.J., Das, D.K., Ray, D.S., Kuszynski, C.A., Joshi, S.S., Pruess, H.G., (2000). Free Radicals and Grape seed proanthocyanidin extract: importance in human health and diseases Prevention. *Toxicology, 148,* 187-197.

Bagchi, D., Sen, K.C., Ray, D.S., Das, D.K., Bagchi, M., Preuss, H.G., Vinson, J.A., (2003). Molecular mechanisims of cardioprotection by a novel grape seed proanthocyanidin extract. *Mutation Research, (523-524),* 87-97.

Barlow, M.S., (1990). Toxicological aspects of antioxidants used as food additives. In B.J.F. Hudson, *Food Antioxidants* (pp. 253). New York: Elsevier Science Publishers

Baydar, N.G., Özkan, G., Sağdıç, O., (2003). Total phenolic contents and antibacterial activities of grape (Vitis Vinifera L.) extracts. *Food Control, (15),* 335-339

Becker, M.E., Nissen, L.R., Skbisted, L.H., (2004). Antioxidant evaluation protocols: food quality or health effects. *European Food Research Technology (219),* 561-571.

Benzie, I.F.F. & Szeto, Y.T., (1999). Total antioxidant capacity of teas by the ferric reducing antioxidant power assay. *Journal of Agriculture and Food Chemistry, (47),* 633-636.

Bonilla F., Mayen M., Merida J., Medina M., (1999). Extraction of phenolic compounds from red grape marc use as food lipid antioxidants. *Food Chemistry* (66), 209-215

Bors, W., Michael, C., Stettmaier, K. Flavonoids and their free radical reactions. GSF Research Center, Neuherberg, Germany.
http://www.medicine.uiowa.edu/frrb/VirtualSchool/Bors-Flavonoids.pdf
(Accessed December, 2005)

Caillet, S., Salmiéri, S., Lacroix, M., (2006). Evaluation of free radical scavenging properties of commercial grape phenol extracts by a fast colorimetric method. *Food Chemistry (95)*, 1-8.

Cho, Y.J., Hong, J.Y., Chun, H.S., Lee, S.K., Min, H.Y., (2005). Ultrasonication assisted extraction of resveratrol from grapes. *Journal of Food Engineering,*

Drapper, N.R., & Smith, H., (1982). *Applied Regression Analysis*, New York: Wiley.

Encinar, J.M., Beltran, F.J., Ramiro, A., Gonzales, J.F., (1998). Pyrolysis/ Gasification of agricultural residues by CO_2 in the presence of different additives: influence of variables. *Fuel Processing Technology, 55,* 219-233.

ETS Laboratories, Technical Bulletin. Grape extracts for monitoring phenolics. http://www.etslabs.com/pagetemplate/blank.asp?pageid=303 (Accessed April 2005)

Folin, O. & Ciocalteu, V., (1927). On tyrosine and tryptophane determinations in proteins. *The Journal of Biological Chemistry, 123 (2),* 627-650

Frankel, E.N., & Meyer, A.S., (2000). Review: The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants, *Journal of the Science of Food and Agriculture, (80)*, 1925-1941.

Frankel, E.N., Waterhouse, A.L., Tussedre, P.L., (1995). Principle phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low-density lipoprotein. *Journal of Agriculture and Food Chemistry, (43),* 890-894.

Frankel, E.N., (2005). *Lipid Oxidation*. 2nd Edition, Bridgwater: Oily Press.

Fuchs, F. J., (1999). Ultrasonic cleaning: fundamental theory and application. Applications Engineering, Blackstone-Ney Ultrasonics Inc., Jamestown, New York, USA.

Fuleki, T. & Silva, R.D.J.M., (1997). Catechin and procyanidin composition of seeds from grape cultivars grown in Ontario. *Journal of Agriculture and Food Chemistry, (45),* 1156-1160.

Gonzales, M.C. & Escarpa, A., (2001). Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. *Analytica Chimica Acta, (427),* 119-127.

Hagerman, A.E., Riedl, K.M., Jones, G.A., Sovik, K.N., Ritchard, N.T., Hartzfeld, P.W., Riechel, T.L., (1998). High molecular weight plant polyphenolics (tannins) as biological antioxidants. *Journal of Agriculture and Food Chemistry, 46,* 1887-1892

Harbers H., (1994). Ash Analysis. In S.S. Nielsen, *Introduction to Chemical Analysis of Foods* (pp. 113-114). New York: Chapman and Hall

Havsteen, B. H., (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics (96),* 67-202.

Jayapraksha, G.K., Selvi, T., Sakariah, K.K., (2003). Antibacterial and antioxidant activities of grape (Vitis vinifera) seed extracts. *Food Research International, (36),* 117-122.

Kandaswami, C. & Middleton, E., (1994). Free Radical Scavenging and antioxidant activity of plant flavonoids. *Advances in Exp. Medical Biology,* (*366)* 351-361.

Katalinic, V., Milos, M., Kulisic, T., Jukic, M., (2006). Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols. *Food Chemistry (94),* 550-557.

Keil, F. J., & Swamy, K. M., (1999). Reactors for sonochemical engineering – presentstatus. *Reviews in Chemical Engineering*, 15 (2), 85-155.

Luque-Garcia, J.L. & Luque de Castro, M.D., (2003). Ultrasound: a powerful tool for leaching. *Trends in Analytical Chemistry, 22* (1), 41-47.

Mason, T. J., (1998). Power ultrasound in food processing – the way forward. In: M. J. W. Povey & T. J. Mason**,** *Ultrasound in Food Processing* (pp 104-124). Glasgow (UK): Blackie Academic & Professional,

Milic, B.L.J., Djilas, S.M., Canadanovic-Brunet J.M., (1998). Antioxidative activity of phenolic compounds on metal-ion breakdown of lipid peroxidation system. *Food Chemistry ,62*, 443-447

Moure, A., Cruz, J.M., Franco, D., Dominguez, J.M., Siniero, J., Dominguez, H., Nunez, M.J., Parajo, J.C., (2001). Natural antioxidants from residual sources. *Food Chemistry (72),* 145-147

Myers, R.H. &. Montgomery, D.C., (1995) *Response Surface Methodology: Process and Product Optimization Using Designed Experiment*s. New York: Wiley.

Nakamura, Y., Tsuji, S., Tonogai, Y., (2003). Analysis of proanthocyanidins in grape seed extracts, health foods and grape seed oil. *Journal of Health Science, 49* (1), 45-54.

Negro, C., Tommasi, L., Miceli, A., (2003). Phenolic compounds and antioxidant activity from red grape marc extracts. *Bioresource Technology (87),* 41-44.

Palma, M., & Taylor, L.T., (1999). Extraction of polyphenolic compounds from grape seeds with near critical carbon dioxide. *Journal of Chromatography A, (849),* 117-124

Pan, X., Niu, G., Liu H., (2003). Microwave-assisted extraction of tea polyhenols and tea caffeine from green tea leaves. *Chemical Engineering and Processing (42),* 129-133.

Pinelo, M., Fabbro, P.D., Manzocco, L., Nunez, M.J., Nicoli, M.C., (2005). Optimization of continuous extraction from Vitis vinifera by products. *Food Chemistry (92),* 109-117.

Polyphenolics Inc., Technical Publication-I.
http://www.polyphenolics.com/pdf/VinoxTechnicalPublication1.pdf
(Accessed May 2005)

Pszczola, D.E., (2001). Antioxidants: From preserving food quality to quality of life. *Food Technology,* 55 (6), 51-59.

Punchard, N. A. & Kelly, F.J., (1996). Free Radicals: A practical approach. New York: Oxford University Press.

Revilla, E., Alonso, E., Kovac, V., (1997). The content of catechins and procyanidins in grapes and wines as affected by agro-ecological factors and technological practices, American Chemical Society, Washington DC, pp: 69-80

Robards, K., Antolovich, M., Prenzler, P., Ryan, D., (2000). Sample Preparation in the determination of phenolic compounds in fruits. *Analyst (125),* 989-1009.

Romandhe, M., & Gourdon, C., (2002). Investigation in solid-liquid extraction: influence of ultrasound. *Chemical Engineering Journal, 87*, 11-19

Rossel, J.B., & Kochhar, S.P., (1990). Detection, estimation and evaluation of antioxidants in food systems. In B.J.F. Hudson, *Food Antioxidants* (pp. 20). New York: Elsevier Science Publishers

Saito, M., Hosoyama, H., Ariga, T., Kataoka, S., Yamaji, N., (1998). Antiulcer activity of grape seed extract and procyanidins. *Journal of Agricultural and Food Chemistry, 46(4)*, 1460-1464.

Shi, J., Yu, J., Pohorly, J.E., Kakuda, Y., (2003). Polyphenolics in grape seeds-Biochemistry and Functionality. *Journal of Medicinal Food, 6*(4), 291-299.

Singleton, V.L., & Rossi, J.A. Jr., (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid Reagents, *American Journal of Enology and Viticulture, 16* 144-158.

Singleton, V.L., (1980). Grape and wine phenolics: backgrounds and prospects. In Proceedings of the University of California, Davis, Grape and Wine Centenary Symposium, University of California, Davis, 215-227,

Slinkard, K. & Singleton, V. L., (1977). Total Phenol Analysis: Automation and Comparison with Manual Methods. *American Journal of Enology and Viticulture* (*28),* 49-55.

Suslick, K.S., (1994). The year of book of science and future, Encyclopedia Britannica (pp.138)

Thompson, D., (1982). Response Surface Experimentation. *Journal of Food Processing and Preservation (6),* 155-158.

Vinatoru, M., Toma, M., Radu, O., Filip, P.I., Lazurca, D., Mason, T.J., (1997). The use of ultrasound for the extraction of bioactive principles from plant materials. *Ultrasonics Sonochemistry, 4*, 135-139

Waterhouse, A., (2001). Folin-Ciocalteu Micro Method for Total Phenol in Wine, Department of Viticulture and Enology University of California, Davis

Yamaguchi, F., Yoshimura, Y., Nakazawa, H., Ariga, T., (1999). Free radical scavenging activity of grape seed extract and antioxidants by electron spin resonance spectrometry in an H_2O_2/NaOH/DMSO system. *Journal of Agricultural and Food Chemistry, 47*, 2544-2548.

Yen, G.C. & Duh, P.D., (1993). Antioxidative properties of methanolic extracts from Peanut Hulls. *Journal of the American Oil Chemistry Society (70),* 383-386.

Yılmaz, Y. & Toledo, R.T., (2006). Oxygen absorbance capacities of grape/wine industry by products and effect of solvent type on extraction of grape seed polyphenols. *Journal of Food Composition and Analysis, 19*(1), 41-48.

APPENDIX

A1 PROCEDURES

A1.1 MOISTURE CONTENT

The moisture content of raw material was determined by applied the following procedure.

1. A glass dish and its lid were dried in the oven to constant weight.
2. The dish was transferred to a desiccator to cool down to the room temperature and its weight was recorded as w_1.
3. Approximately 3 grams of pomace was weighted (w_p) and put in the dish, and it was kept into oven at 105°C.
4. The dish was kept in the oven for 2 hours.
5. After two hours, the dish was covered with the lid while inside the oven.
6. The dish was placed into desiccator to cool down to the room temperature and the final weight of the dish and its content was measured and recorded as w_2.

The moisture content is calculated on the 24 hours dried basis by following equation.

$$Moisture\ Content, \% = \frac{w_p - (w_2 - w_1)}{w_p} * 100 \qquad (5)$$

w_1 : Weight of empty glass dish, g

w_2 : Weight of the glass dish and its contents, g

w_p : Weight of the sample, wet basis,

A1.2 SIEVE ANALYSIS

1. Approximately 200 grams of raw material (w_p) was ground in a cutting mill.
2. Sieves were placed in a sieve shaker in a decreasing aperture size order and the sieves were shaken for 10 minutes.
3. Sieve mesh was operated 10 minutes.
4. The weight of sample on each sieve was measured and recorded as w_{p_i} .

The weight fraction of sample with corresponding size ranges is calculated by using following equation.

$$Weight\ Fraction, \% = \frac{w_{p_i}}{w_p} * 100 \qquad (7)$$

w_p : Total weight of sample, g

w_{p_i} : Weight of sample on each sieve after operation,

A1.3 ASH CONTENT

Ash content of raw pomace was determined by following procedure.

1. Porcelain crucibles and lids were preheated at around 600°C overnight in muffle furnace.
2. The crucible was then transferred into a desiccator to cool down to the room temperature and its weight was recorded as w_{c1}.
3. Approximately 2 grams of pomace was weighted (w_p) in this crucible and placed into the muffle furnace at 600°C.
4. The crucible was kept in the oven for 4 hours.
5. After 4 hours, the temperature of muffle was gradually decreased and crucible was transferred to the desiccator.
6. The crucible was covered with the lid and cooled down to the room temperature.
7. The final weight of crucible and its content was recorded as w_{c2}.

The ash content of merlot type grape pomace is calculated by using the following equation.

$$Ash\ Content, \% = \frac{w_{c2} - w_{c1}}{w_p} * 100 \qquad (8)$$

w_{c1}: Weight of the empty crucible, g

w_{c2}: Weight of the crucible and its contents, g

w_p: Weight of the sample, g

A1.4 ULTRASONIC EXTRACTION

The ultrasound assisted extraction procedure is as follows:

1. Erlenmayer flask with volume of 100 mL was cleaned and dried in the oven, and placed in the desiccator to cool to the room temperature.
2. Specific amount of grape pomace (5 g) is weighted inside the flasks, w_p .
3. Specific amount of solvent, v_s , was added to the flask
4. The top of the flask was covered with cork stopper and equipped with thermometer.
5. The ultrasonic bath was filled with the distilled water, 4 cm below the top.
6. Flask was placed was placed inside the bath such that the solvent level inside the flask was 2 cm below the level of water in the bath.
7. The sonication was carried (ST).

w_p : Weight of the sample, g

v_s : Volume of solvent, mL

ST: Sonication time, min

A1.5 SHAKING WATER BATH EXTRACTION

1. The water bath was filled with deionised water.
2. Temperature was set to specific value (45°C).
3. Immediately after taking the flask from ultrasonic bath, it was first kept in boiling water a few seconds for the contents to reach 45°C.
4. Afterwards, the flask was placed into water bath and the shaker was adjusted to specific rate.
5. The operation was carried out for time, WBT.

WBT: Time of water bath extraction, min

A1.6 SOLID-LIQUID SEPARATION

The following way of centrifugation was used.

1. A solution of 10 % TCA was prepared by dissolving 10 grams of TCA in ultra pure water (Milli Q, Millipore).
2. Immediately after the extraction, two samples (each approximately 15 mL) were taken from the flask and put into test tubes.
3. 0.7 mL of TCA solution was added to each tube.
4. The tubes were placed into centrifuge.
5. The centrifugation was carried out 15 minutes at 1750g.

A1.7 ANALYSIS of TOTAL PHENOLICS CONTENT

The following procedure was used to determine the total phenolics content of extracts.

First, the gallic acid and the sodium carbonate stock solutions were prepared.

Gallic Acid Stock Solution: In a 100 mL volumetric flask, 0.500 g of dry gallic acid is dissolved in 10 mL of ethanol and completed to 100 mL with deionised water. To store, keep closed in a refrigerator up to two weeks.

Sodium Carbonate Solution: 15 grams of sodium carbonate was dissolved in 120 mL of deionised water while heated on magnetic stirrer until water boils. After cooling to room temperature, a few sodium carbonate crystals were added to the solution. The solution was kept for 24 hours. Afterwards, the solution was filtered if required, and 30 mL of deionised water was added to bring the total volume 150 mL. This would make the solution as 10 %.

Analysis Procedure:

1. 20 µL of sample was taken into test tube immediately after the centrifugation.
2. Add 6.5 mL of deionised water and apply 10 seconds of vortex mixing.
3. Add 500 µL of non-diluted Folin's Reagent to this mixture and again apply 10 seconds of vortex mixing
4. Then, keep the solution for 8 minutes at room conditions
5. At the end of 8 minutes, add 3 mL of 10 % sodium carbonate solution to the mixture and once more apply 10 seconds of vortex mixing
6. Then, place the mixture into shaking water bath at 40°C and keep there 30 minutes for reaction

7. At the end of 30 minutes of reaction, read the absorbance against the blank solution, which includes all reagents except the sample, at 765 nm.

Calibration Curve Preparation: The calibration curve was prepared using the gallic acid (phenol) stock solution. In order to prepare the calibration curve,

1. Add 0, 1, 2, 3, 5, 10 mL of the gallic acid stock solution, described above, into 100 mL bakers and dilute wit water to 100 mL with deionised water
2. These solutions will have phenol concentrations of 0, 50, 100,150, 250, and 500 mg/L gallic acid.
3. Take 100 µL of each solution and put into test tube
4. Add 6.5 mL of deionised water and apply 10 seconds of vortex mixing.
5. Add 500 µL of non-diluted Folin's Reagent to this mixture and again apply 10 seconds of vortex mixing.
6. Then, keep the solution for 8 minutes at room conditions.
7. At the end of 8 minutes, add 3 mL of 10 % sodium carbonate solution to the mixture and once more apply 10 seconds of vortex mixing.
8. Then, place the mixture into shaking water bath at 40°C and keep there 30 minutes for reaction
9. At the end of 30 minutes of reaction, read the absorbance against blank a blank solution at 765 nm.

In the analysis of extracts 20 µL of sample was used in order to be in the linear range in spectrophotometer readings. However, 100 µL of gallic acid solutions was used in calibration curve preparation. The results obtained from the calibration curve were therefore multiplied by a factor of 5. Absorbance data for calibration and the calibration curve were presented on Table-5 and Figure-13, respectively.

Table-8: Absorbance Data for Calibration Curve

CONCENTRATION (mg/L GAE)	ABSORBANCE UNITS (Average)
0	0
50	0.0656
100	0.1241
150	0.1942
250	0.3102
500	0.5753

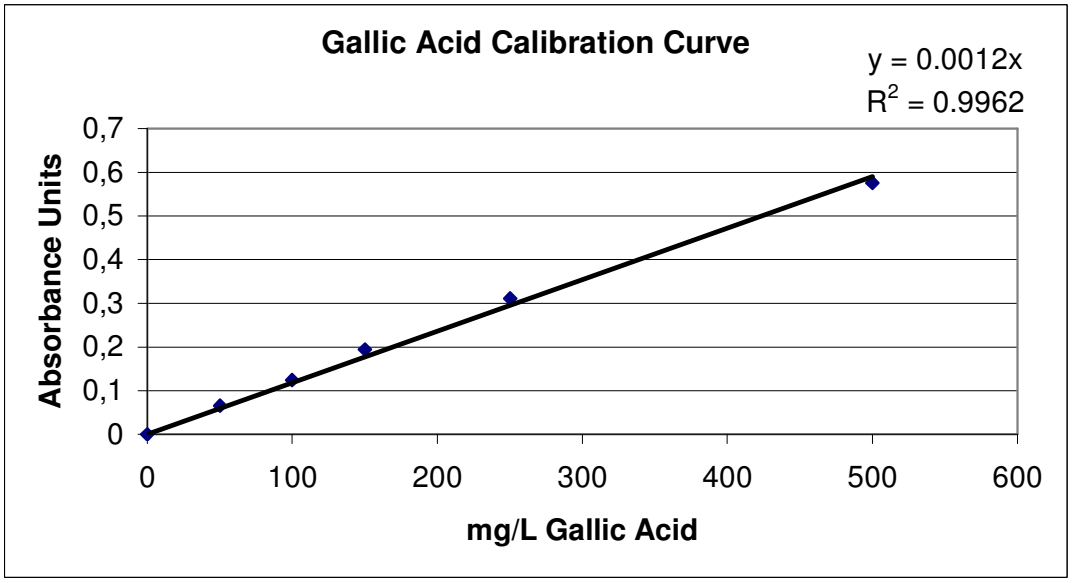

Figure-13: Gallic Acid Calibration Curve

A2 EXPERIMENTAL DATA and RESULTS

Table-9: Experimental Data and Results

RUN	ST (min)	WBT (min)	SC (% v/v EtOH)	SOLVENT (mL)	SAMPLE (g)	GAE g/L
1	14	28	70	100	5.042	2.10
2	14	12	70	100	5.038	1.88
3	14	28	30	100	5.024	1.88
4	14	12	30	100	5.044	1.80
5	6	28	70	100	5.012	1.48
6	6	12	70	100	5.014	1.30
7	6	28	30	100	5.028	1.40
8	6	12	30	100	5.014	2.36
9	0	20	50	100	5.026	1.52
10	20	20	50	100	5.084	2.30
11	10	0	50	100	5.002	0.90
12	10	40	50	100	5.088	1.86
13	10	20	0	100	5.012	0.32
14	10	20	100	100	5.062	0.84
15	10	20	50	100	5.064	1.70
16	10	20	50	100	5.072	1.90
17	10	20	50	100	5.044	1.82
18	10	20	50	100	5.032	1.42
19	10	20	50	100	5.044	1.56
20	10	20	50	100	5.052	1.64
21	10	20	50	100	5.048	1.56
22	10	20	50	100	5.074	1.94
23	10	20	50	100	5.026	1.68

Table-10: Absorbance Readings

RUN	ST (min)	WBT (min)	SC (% v/v EtOH)	Temperature [(a)] (°C)	Absorbance Readings
1	14	28	70	40	0.5052
2	14	12	70	39	0.4524
3	14	28	30	36	0.4522
4	14	12	30	36	0.4296
5	6	28	70	36	0.3561
6	6	12	70	35	0.3054
7	6	28	30	35	0.3371
8	6	12	30	36	0.5660
9	0	20	50	-	0.3671
10	20	20	50	34	0.5447
11	10	0	50	34	0.2144
12	10	40	50	34	0.4459
13	10	20	0	33	0.3865
14	10	20	100	38	0.2005
15	10	20	50	38	0.4158
16	10	20	50	36	0.4546
17	10	20	50	35	0.4384
18	10	20	50	34	0.3415
19	10	20	50	36	0.3754
20	10	20	50	36	0.3931
21	10	20	50	35	0.3720
22	10	20	50	35	0.4681
23	10	20	50	34	0.4037

[(a)] Final temperature values during ultrasonication

A3 STATISTICAL TREATMENTS

A3.1 REPRODUCIBILITY

The results of reproducibility experiments in increasing order were as follows: 1.42, 1.56, 1.56, 1.64, 1.68, 1.70, 1.82, 1.90, 1.94 g/L GAE. The standard deviation (s) of the data was 0.17 (s).

Q-Test Application: For 96 % Confidence Level and n = 9, Q = 0.51

$$Q_1 = \frac{x_2 - x_1}{x_n - x_1} \qquad Q_n = \frac{x_n - x_{n-1}}{x_n - x_1} \tag{9}$$

$$Q_1 = \frac{1.56 - 1.42}{1.94 - 1.42} = 0.27$$

$$Q_2 = \frac{1.94 - 1.90}{1.94 - 1.42} = 0.076$$

Since both values were smaller than 0.51, there was no need to reject of any data for 96 % confidence.

t-Score Application For True Mean: For 95 % confidence level and degree of freedom, df = n-1 = 8, t = 2.31, then

$$\mu = \bar{x} \mp \frac{s}{\sqrt{n}} * t \tag{10}$$

$$\mu = 1.70 \pm \frac{0.17}{3} * 2.31$$

$$\mu = 1.70 \pm 0.13 \ g/L \ GAE$$

Checking Systematic Error: In order to decide whether the difference between true mean and average is significant, t value is calculated by using Eqn. 9, and if exceeds the certain critical value, in this case 2.31, this indicates the existence of systematic error.

$$t = \frac{0.13 * 3}{0.17} = 2.28$$

Calculated t-value is smaller than the critical value. Therefore, there is no evidence of systematic error.

A3.2 STATISTICAL ANALYSIS of MODEL

Lack of Fit Test: This test is applied in order to determine the adequacy of the model. F_{LOF} can be defined as the ratio of mean square of lack of fit (MSL) to the mean square of pure error (MSE).

In order to evaluate the F_{LOF}, first sum of squares of lack of fit (LSS) and then MSL should be determined. The residual sum of square (RSS) is equal to summation of pure error sum of square (ESS) and LSS.

$$RSS = ESS + LSS \qquad (11)$$

Similarly, degree of freedom (df) of RSS is equal to summation of pure error degree of freedom and lack of fit degree of freedom.

$$Rdf = Edf + Ldf \qquad (12)$$

Sum of squares and degree of freedom of residual was obtained from Statgraphics©, as indicated in Table-10. Sum of squares and degree of freedom of pure error was obtained from the reproducibility experiments. The ESS was evaluated by using Eqn (13)

$$ESS = \sum_{i=1}^{9} y_i^2 - \bar{y} \qquad (13)$$

In the formula, y_i denotes each recovery value found by reproducibility experiments and $\bar{y}$ denotes the average value. Therefore, ESS was evaluated as 748. On the other hand, degree of freedom of pure error is equal to

$$Edf = n - 1 \qquad (14)$$

Here, n denotes the number of replicated observations, which was 9, in this study. Therefore, Edf was equal to 8. Mean square for pure error can be found by ESS and Edf values as follows,

$$MSE = \frac{ESS}{Edf} \qquad (15)$$

$$MSE = \frac{748}{8}$$
$$MSE = 93.5$$

By subtracting ESS from RSS, which was evaluated as 1536.48 by using Statgraphics, LSS can be evaluated.

$$LSS = 1536.48 - 748$$
$$LSS = 789$$

The degree of freedom of lack of fit can be evaluated by subtracting pure error degree of freedom from residual degree of freedom, which was found as 13.

$$Ldf = 13 - 8$$
$$Ldf = 5$$

Mean square of lack of fit (MSL) can be evaluated by as follows,

$$MSL = \frac{LSS}{Ldf} \qquad (16)$$

$$MSL = \frac{789}{5}$$
$$MSL = 157.8$$

By using MSE and MSL, F_{LOF} can be evaluated as follows,

$$F_{LOF} = \frac{MSL}{MSE} \qquad (17)$$

$$F_{LOF} = \frac{157.8}{93.5} = 1.68$$

If lack of fit of the model is smaller than tabulated F value based on Ldf and Edf, then model is accepted as adequate. The tabulated F value is 3.69 at 95 % confidence level. Therefore, on the basis of this test there is no reason to doubt the adequacy of the model. However, the developed model was purely empirical. The fact that the model passes all tests does not mean that it is the correct model. It is only reasonable one, which has not been found inadequate by the data. Therefore, not only the empirical model alone, but also the physical phenomena of the experiment system should be taken into consideration in the assessment of the results.

A4 ULTRASONIC BATH and PROBE SYSTEMS

In Figure-14 below, a schematic of ultrasonic bath and its major components are shown.

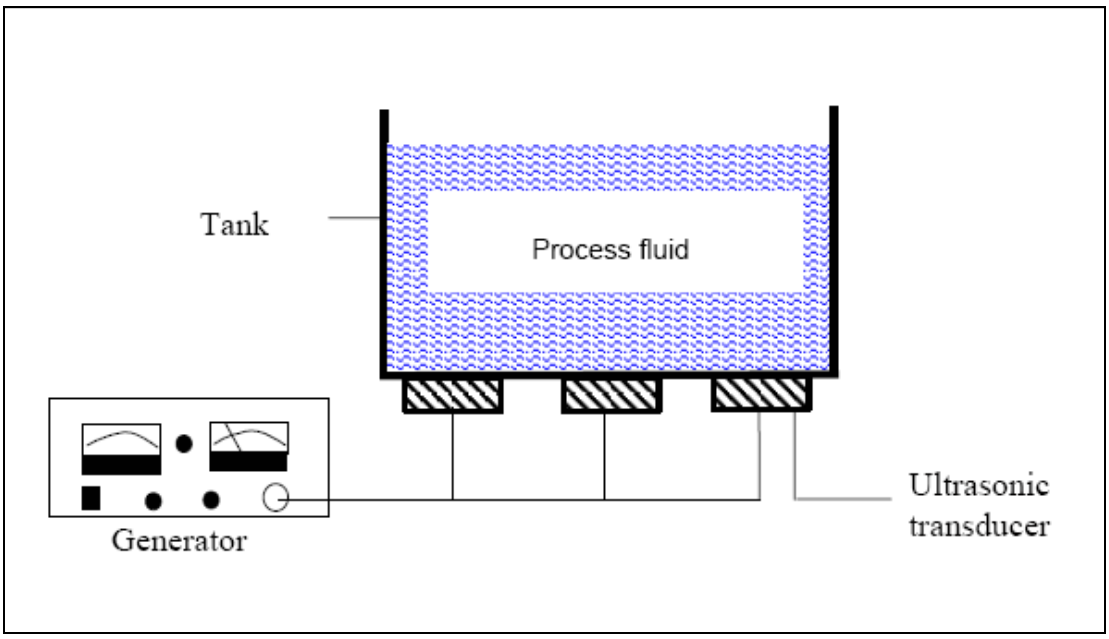

Figure-14: Schematic Ultrasonic Bath [Adapted from Fuchs (1999)]

As seen, tank, power generator and ultrasonic transducer are the main parts of the bath. The major function of power generator is to convert a standard electrical frequency (5-60 Hz) into alternating frequency (over 20 kHz). Ultrasonic transducers are bonded to base of ultrasonic bath. They can be magnetostrictive type or piezoelectric type. The task of the transducers is transferring the electrical frequency into processing fluid as mechanical vibrations. In Figure-15, schematic of magnetorestrictive transducer is indicated. Application of electric current to coil creates a magnetic field that results in the reduction of dimensions of core. Switching off the current causes to return of core to its original dimensions. Continuous changes in dimensions create mechanical vibrations in processing fluid. [Fuchs, 1999]

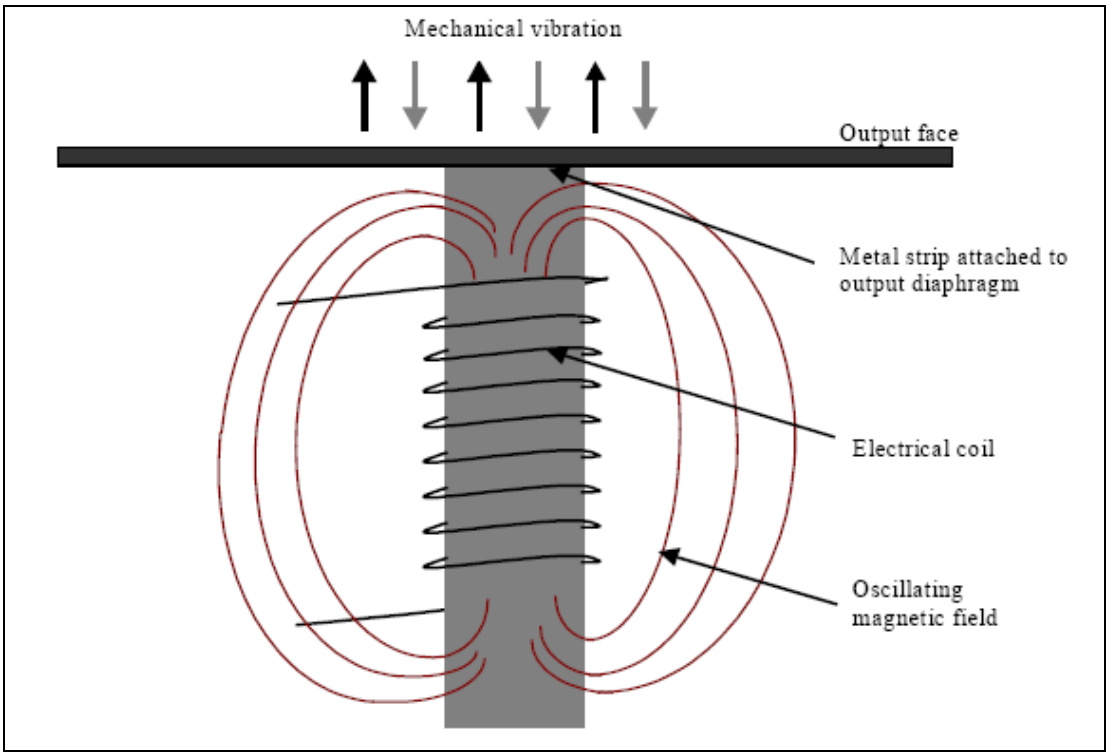

Figure-15: Magnetostrictive transducer [Adapted from Fuchs (1999)]

In Figure-16, schematic of piezoelectric transducer are shown. This type of transducers is the most commonly used type of transducers. In their construction, piezoelectric ceramics are utilized, which will expand and contract in an alternating electric field, leading to pressure waves transmitting through the medium [Fuchs, 1999].

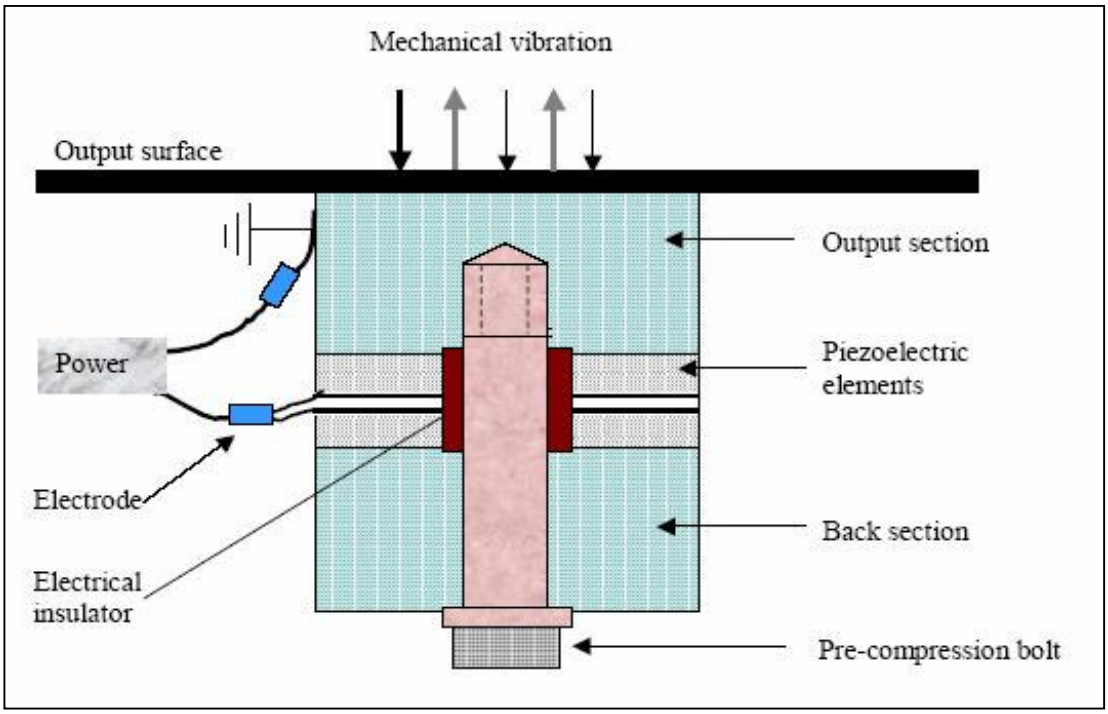

Figure-16: Piezoelectric Transducer [Adapted from Fuchs (1999)]

On the other hand, ultrasonic probes have the advantage over ultrasonic baths in the way that they focus energy on a localized sample zone and so providing more efficient cavitation in the liquid [Luque-Garcia & Luque de Castro, 2003]. Schematic of ultrasonic probe is depicted in Figure-17. The horns must be designed to resonate at the same frequency as the transducer that drives it. It is important to obtain the correct amplitude of movement of the horn tips, which is dependent on its shape and dimensions [Keil & Swamy, 1999].

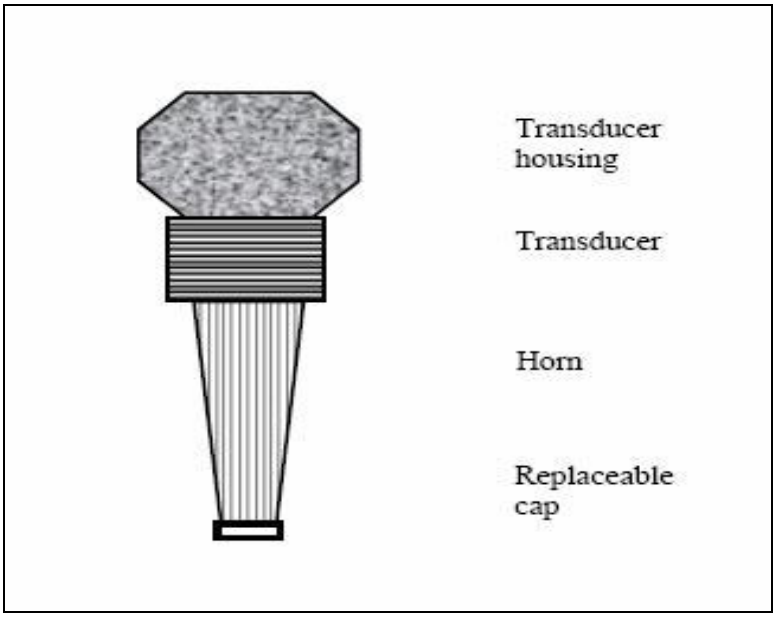

Figure-17: Schematic of Ultrasonic Probe [Adapted from Mason (1998)]