ULTRASOUND ASSISTED AND SUPERCRITICAL CARBON DIOXIDE EXTRACTION OF ANTIOXIDANTS FROM ROASTED WHEAT GERM

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ABSTRACT

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This study covers the extraction of antioxidants from wheat germ; which is the byproduct of the flour-milling industry and a rich source of antioxidants; with Ultrasound Assisted (UAE) and Supercritical Carbon Dioxide (SC-CO_2) extractions. Extraction conditions were ultrasonication time (1–11 min), temperature (20–60°C) and ethanol level (5–95%) for UAE, and pressure (148–602 bar), temperature (40–60°C) and time (10–60 min) for SC-CO_2 extraction. The extraction conditions were optimized based on yield (%), total phenolic contents (TPC, mg GAE/g extract) and antioxidant activities (AA, mg scavenged $DPPH^{\cdot}$/g extract) of the extracts, using Central Composite Rotatable Design. Total tocopherol contents (TTC) of the extracts were determined, as well.

UAE (at 60°C) with low ethanol level (~5-30%) and short times (1-3 min) provided protein rich extracts with high yield, medium TPC and AA. On the other hand, with high ethanol level (~90%) and long times (6-11 min), waxy structured extracts with low yield but high TPC and AA were obtained.

SC-CO_2 extraction at 442 bar, 40ºC and 48 min. enabled almost 100% recovery of wheat germ oil (9% yield) but TPC and AA of the extracts were low. On the contrary, the extracts obtained at lower pressures (~150bar) and shorter times (~10 min) at 50-60ºC had high TPC and AA since the oil yield was low. However, TPC and AA of these extracts were only half of those extracted by UAE. Maximum tocopherol (7.142 mg tocopherol/g extract) extraction was achieved at 240 bar, 56ºC for 20 min. Both of the methods extracted high amounts of tocopherols from roasted wheat germ (SC-CO_2 extraction; 0.31 mg tocopherol/g germ, UAE; 0.33 mg tocopherol/g germ) but TTC of the extracts obtained by SC-CO_2 extraction was superior compared to 1.170 mg tocopherol/g extract obtained by UAE at 9 min, 58ºC and 95% ethanol level.

All these extracts with different characteristics have potential uses in cosmetic and food industry depending on the targeted specific application.

Keywords: Wheat Germ, Ultrasound Assisted Extraction, Supercritical Carbon Dioxide Extraction, Total Phenolic Content, Antioxidant Activity.

ÖZ

KAVRULMUŞ BUĞDAY RÜŞEYMİNDEN ULTRASON DESTEĞİ
VE SÜPERKRİTİK KARBON DİOKSİT KULLANIMIYLA
ANTİOKSİDAN OZÜTLENMESİ

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Bu çalışma, un sanayisinin yan ürünü ve zengin bir antioksidan kaynağı olan buğday rüşeyminden ultrason destekli (UAE) ve süperkritik karbondioksit (SC-CO_2) kullanımıyla antioksidan özütlenmesini kapsamaktadır. Özütleme koşulları UAE için ultrasonikasyon süresi (1–11 dk.), sıcaklık (20–60°C) ve etanol seviyesiydi (5–95%) ve SC-CO_2 özütleme için basınç (148–602 bar), sıcaklık (40–60ºC) ve süre (10–60dk.)olup, özütleme koşullarının optimizasyonu, verime (%) özütlerin toplam fenolik içerikleri (TPC, mg GAE/g özüt) ve antioksidan aktivitelerine (AA, mg indirgenmiş $DPPH^{\cdot}$/g özüt) göre merkezi kompozit dizaynı ile optimize edilmiştir. Bunun yanı sıra özütlerin toplam tokoferol içerikleri de (TTC) belirlenmiştir.

Düşük etanol seviyesiyle (~5–30%) ve kısa sürelerde (1–3 min) UAE (60°C) protein açısından zengin yüksek verimli orta derecede TPC ve AA'sı olan özütler sağlamıştır. Öte yandan yüksek etanol seviyesiyle (~90%) ve uzun sürelerde (6–

11 min) düşük verimli fakat yüksek TPC ve AA'sı olan vakslı özütler elde edilmiştir.

442 bar, 40ºC ve 48 dakika SC-CO_2 kullanımıyla özütleme buğday rüşeymi yağının takriben %100'ünün (%9 randıman) kazanılmasını mümkün kılmış ancak bu koşullarda düşük TPC ve AA değerli özütler elde edilmiştir. Aksine, düşük basınçlarda (~150bar), 50–60ºC sıcaklıkta ve kısa sürelerde (~10 dk.) elde edilen özütlerde TPC ve AA konsantrasyonları yüksek elde edilmiştir. Ne var ki, bu değerler UAE ile özütlenenlerin ancak yarısı kadardır. Maksimum tokoferol (7.142 mg tokoferol/g özüt, 0.31 mg tokoferol/g ruşeym) özütlenmesi 240 bar, 56ºC, 20 dakikada sağlanmıştır. Her iki metotta kavrulmuş buğday rüşeyminden yüksek miktarlarda tokoferol özütlemiştir (SC-CO_2 özütleme; 0.31 mg tokoferol/g rüşeym, UAE; 0.33 mg tokoferol/g rüşeym) fakat SC-CO_2 özütleme ile elde edilen özütlerin TTC miktarları, UAE ile 9 dk., 58ºC ve 95% etanol seviyesinde elde edilen "1.170 mg tokoferol/g özüt" ile karşılaştırıldığında çok üstündür.

Farklı karakterdeki bu özütlerin, hedeflenen özel uygulamaya göre kozmetik ve gıda sanayisinde muhtemel kullanım alanları mevcuttur.

Keywords: Buğday Rüşeymi, Ultrason Destekli Özütleme, Süperkritik Karbon Dioksit Özütleme, Toplam Fenol Miktarı, Antioksidan Aktivitesi.

To the memory of my beloved uncle DR. İSMAİL KAVUŞTU

(1956-2006)

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ABBREVIATIONS

OFR	Oxygen Free Radicals
PUFA	Polyunsaturated Fatty Acid
TSE	Traditional Solvent Extraction
EtOH	Ethanol
GAE	Gallic Acid Equivalents
CO_2	Carbon dioxide
$DPPH^{\cdot}$	2, 2-diphenyl-1-picrylhydrazyl radical
E %	Specified Ethanol Level (%) in solvent for each UAE.
T_{US}	Specified Ultrasonication temperature for each UAE.
T_{US}	Specified Ultrasonication time for each UAE
$w_{f.i}$	Weight of empty rotatory dryer collecting flask
w_f	Weight of rotatory dryer flask was recorded after cooling
E	Amount of extract for each run
$E.Y_u$ %	Extraction Yield for UAE.
w_g	Weight of roasted wheat germ, used in SC-CO_2 extraction.
w_{ti}	Weight of empty collected tube, used in SC-CO_2 extraction.
w_{tf}	Weight of extract and tube, used in SC-CO_2 extraction.
E_{SCO2}	Amount of extract of SC-CO_2 extraction.
$E.Y_{SCO2}$	Extraction Yield of SC-CO2 extraction
TCA	Trichloroacetic Acid
TPC	Total Phenolic Content
AA	Antioxidant Activity

CHAPTER 1

INTRODUCTION

1.1 WHEAT

Wheat (*Triticum spp.*) is a domesticated grass from the Levant that is cultivated worldwide. "The Levant" is a geographical term historically referring to a large area in the Middle East south of the Taurus Mountains, bounded by the Mediterranean Sea on the west and by the northern Arabian Desert and Upper Mesopotamia to the east (Wikipedia, 2007). Wheat grain is an important cereal grinded to make flour and fermented to make beer, alcohol, vodka or biofuel. The cultivation of wheat is thought to have had its origin in the Fertile Crescent of Middle East, carbonized remains of wheat grains in baked clay have been found in Neolithic site of Jarno in northern Iraq having an estimated radiocarbon date of 6700 B.C (Inglett, 1974). Also Mangelsdorf suggested that wheat had its origin in the Caucasus-Turkey-Iraq area (Huges et al., 1957).

1.1.1 CLASSIFICATION OF WHEAT

The genus Tricitum includes the wild and domesticated species usually thought of as wheat. Some of the major cultivated species of wheat are (Wikipedia, 2007);

- **Common wheat** or Bread wheat — (*Triticum aestivum*): A hexaploid species that is the most widely cultivated in the world. This species contains a high

percentage of gluten and is used to make bread and fine cakes.

- **Durum** — (*Triticum durum*): The only tetraploid form of wheat widely used today, and the second most widely cultivated wheat. The hardest-kernelled wheat is durum; its flour is used in the manufacture of macaroni, spaghetti, and other pasta products.
- **Einkorn** — (*Triticum monococcum*): A diploid species with wild and cultivated variants. Domesticated at the same time as emmer wheat, but never reached the same importance.
- **Emmer** — (*Triticum dicoccon*): A tetraploid species, cultivated in ancient times but no longer in widespread use.
- **Spelt** — (*Triticum spelta*): Another hexaploid species cultivated in limited quantities.

Within a species, wheat cultivars are classified in terms of growing season, such as winter wheat vs. spring wheat, by gluten content, such as hard wheat (high protein content) vs. soft wheat (high starch content), or by grain color (red, white or amber) (Wikipedia, 2007). Hard wheats are usually used to make bread because of their high gluten and protein content. In patisseries soft wheats are used.

Classification of wheat in Turkey differs from American Classification. There are nine classes of wheat specified due to region of planting, hardness, color and shape of kernels. General classification in Turkey is; Milling wheats (Anatolian Hard White, Anatolian Red White, Semi Hard Red, Semi Hard White Feed Wheat, Others) and Durum wheats (Anatolian, low quality and others).

1.1.2 WHEAT PRODUCTION

Wheat is widely cultivated as a cash crop because it produces a good yield per

unit area, grows well in a temperate climate even with a moderately short growing season. Wheat is harvested somewhere in the world in nearly every month of the year (Pomeranz, 1987). Worldwide production of wheat is seen in Table 1.1.

Table 1.1 World Wheat Production (million ton per year) (FAO, 2007)

CONTINENT / COUNTRY	Year					
	2000	**2001**	**2002**	**2003**	**2004**	**2005**
Africa	14	18	17	22	22	21
Turkey	21	19	19.5	19	21	21
Europe	184	202	212	154	220	208
Asia	255	247	254	245	256	268
World	586	590	575	561	632	631

Wheat is the most produced cereal in Turkey (Table 1.2). Turkey is one of the top ten wheat producers (Table 1.3) in the world with 3% of the world's production.

Table 1.2 Cereal Production in Turkey in 2007 (FAO, 2007)

Cereal	**Production** (million ton)
Wheat	21
Barley	9.5
Maize	4.2
Oats	0.27
Rye	0.27
Rice, paddy	0.6

Table 1.3 World Top Ten Wheat Producers of 2005 (FAO, 2007)

Country	**Production** (million ton)
China	96
India	72
United States	57
Russia	46
France	37
Canada	26
Australia	24
Germany	24
Pakistan	22
Turkey	21

1.1.3 WHEAT KERNEL

The kernel is the seed from which the plant grows. The wheat kernel contains three main parts; endosperm, the outer coat of the kernel (bran) and the sprouting section (wheat germ).

The endosperm makes up 83% of the kernel and contains carbohydrates and B-complex vitamins. Also starch is stored in endosperm. The bran is about 14% of the kernel weight. The bran contains a small amount of protein, large quantities of the three major B vitamins, trace minerals and indigestible cellulose material (dietary fiber). Bran consists of layers epidermis, hypodermis, cross cells, tube cells, seed coat (testa), nucellar tissue and aleurone. The four outer layers (epidermis-hypodermis-cross cells-tube cells) form the outer bran called "pericarp". The inner layer aleurone covers the endosperm. Figure 1.1 shows all layers in order.

The remaining tissue is embryo or "The Germ". It weighs about 2.5% of the

kernel's total weight.

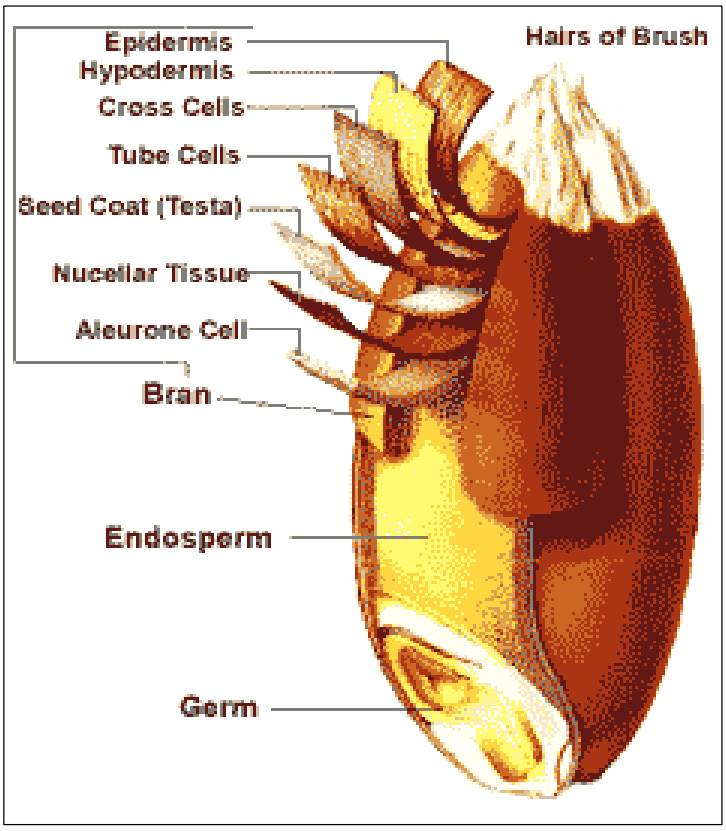

Figure 1.1 Wheat Kernel (Four Leaf, 2008)

1.1.4 WHEAT GERM

Wheat germ is composed of three parts; the embryonic axis, the scutellum and the epiblast. On germination the embryonic axis develops into a seedling, and the scutellum nourishes. Wheat germ is a unique source of highly concentrated nutrients. It offers three times as much protein of high biological value, seven

times as much fat, fifteen times as much sugar, and six times as much as mineral content when compared with flour from the endosperm (Shurpalekar et al., 1977). The composition of wheat germ is given in Table 1.4. Typical energy supplied by wheat germ is 360 kcal (1506 kJ) per 100 grams (NAL, 2008).

Table 1.4 Composition of Wheat Germ (NAL, 2008)

Nutrient	Amount (g/100 grams)
Carbohydrate	51.80
Protein	23.15
Water	11.12
Total lipid (fat)	9.72
Ash	4.21

The presence of large amounts of fats and sugars makes wheat germ highly palatable. In view of its high nutritive value and palatability, wheat germ offers an excellent source of vitamins, proteins and minerals for fortification of food products (Shurpalekar et al., 1977).

Table 1.5 Vitamin B Group and Vitamin E Content of Some Cereals (mg /100 g edible portion) (Belitz and Grosch, 1999)

Food Product	Vitamin B_1	Vitamin B_2	Vitamin B_3	Vitamin E (α-tocopherol)
Wheat Germ	2.01	0.72	3.3	27.6
Oat Flakes	0.59	0.15	0.16	3.7
Rye Flour	0.19	0.11	-	3.4
Corn Flakes	-	-	0.07	0.43
Wheat Flour	0.11	0.08	0.1	2

Wheat germ is the richest source of tocopherols of plant origin (Table 1.5) and also a rich source of niacin and thiamine as well (Table 1.6). Tables 1.7 and 1.8 give the amino acid composition and the mineral composition of wheat germ, respectively.

Table 1.6 Vitamin Composition of the Wheat Germ (NAL, 2008)

Vitamins	Amount (mg/100 grams)
Niacin	6.813
Pantothenic acid	2.257
Thiamin	1.882
Vitamin B6	1.300
Riboflavin	0.499
Folate, total	0.000281

Table 1.7 Wheat Germ Amino Acids (NAL, 2008)

Amino acids	Amount (g/100 grams)
Glutamic acid	3.995
Aspartic acid	2.070
Arginine	1.867
Leucine	1.571
Alanine	1.477
Lysine	1.468
Glycine	1.424
Proline	1.231
Valine	1.198
Serine	1.102
Threonine	0.968
Phenylalanine	0.928
Isoleucine	0.847
Tyrosine	0.704
Histidine	0.643
Cystine	0.458
Methionine	0.456
Tryptophan	0.317

Tocopherols are known to be fat soluble. Since wheat germ is rich in vitamin E (2.76 mg/g germ) than wheat germ oil is rich in vitamin E as well. α-tocopherol content of wheat germ oil is reported to be 1.5 mg/g wheat germ oil (NAL, 2008). This amount is richer than olive oil, sunflower oil, soybean oil and sesame oil (Anon, 1993). Wheat germ oil contains choline (0.2 mg/g germ oil) (NAL, 2008) as well and is rich in polyunsaturated fatty acids (Table 1.9)

Table 1.8 Mineral Composition of the Wheat Germ (NAL, 2008)

Minerals	Amount (mg/100 grams)
Potassium (K)	892
Phosphorus (P)	842
Magnesium (Mg)	239
Calcium (Ca)	39
Manganese (Mn)	13.301
Zinc (Zn)	12.29
Sodium (Na)	12
Iron (Fe)	6.26
Copper (Cu)	0.796
Selenium (Se)	0.0792

Essential Fatty Acids deficiency, particularly ω-3 deficiency is a common health problem. An ideal intake ratio of ω-6 to ω-3 fatty acids is between 1:1 and 4:1, with most people only obtaining a ratio between 10:1 and 25:1 (Wikipedia, 2008). The ratio of ω-6/ ω-3 is 7.7 (Table 1.9) in wheat germ oil, where it is rarely below 10 in most of the food. This adds an additional value to wheat germ oil. Wheat germ oil is used in products such as foods, biological insect control agents, pharmaceuticals and cosmetic formulations and has been shown to reduce plasma and liver cholesterol in animals and to delay aging (Kahlon, 1989).

Table 1.9 Lipid Characterizations of Wheat Germ and Germ Oil

Lipid	Amount	
	g/100 g wheat germ	**g/100 g wheat germ oil**
Fatty acids, total saturated	1.665	18.8
14:0	0.013	0.1
16:0	1.587	16.6
18:0	0.055	0.5
Fatty acids total monounsaturated	1.365	15.1
16:1 undifferentiated	0.034	0.5
18:1 undifferentiated	1.332	14.6
Fatty acids total polyunsaturated	6.010	61.7
18:2 undifferentiated	5.287	54.8
18:3 undifferentiated	0.723	6.9

1.1.4.1 RANCIDITY

The problem of rancidity is greatest in cereal components which have high oil content like wheat germ (Kent and Evers, 1994). It is highly susceptible to rancidity owing to presence of large amounts of unsaturated fats and of oxidative as well as hydrolytic enzymes (Shurpalekar et al., 1977).

From the previous works (Sherwood et al., 1933 & Pearce, 1943), it is known that raw germ on storage develops a rancid flavor and bitter taste in a short time. Analyzed volatiles of over-stored wheat germ were common lipid oxidation products and included medium chain aldehydes, ketones, alcohols, hydrocarbons, acids and 2-pentyl furan (El- Saharty et al., 1998). Rothe (1963) observed that, because of high enzyme activity and unsaturated fat content in fresh germ the organoleptic acceptability is affected adversely within days. During the storage of wheat germ, a loss of tocopherols was reported (Wierzbowski et al., 1966).

Although it has high nutritional value, wheat germ is generally used in animal feed formulations. Poor storage stability has been a restriction for usage of wheat germ as a food ingredient. Germ must be stabilized before it is processed. Different methods examining the effect of decreasing moisture, oil content or enzyme activity on rancidity and storage properties of wheat germ have been studied.

1.1.4.2 STABILIZATION OF WHEAT GERM

Raw germ is highly unstable and deteriorates rapidly within a few days, as it is rich in enzymes, lipase, lipoxidase, and protease. Among those different enzymes present in the germ, lipase is probably the most important enzyme due to its role in the development of hydrolytic rancidity during storage of the germ or products containing germ. Inactivation of lipase assumes paramount importance for improving the shelf life of the germ and germ products (Shurpalekar et al., 1977).

Methods suggested for stabilization of wheat germ includes toasting, defatting and steaming, infrared heating, microwave treatment, lowering the moisture content by different drying methods, antioxidant treatment and packing under vacuum or inert gases (Shurpalekar et al., 1977).

Light toasting of the germ at 120º-130ºC until it attained a light brown color improved germs keeping quality as well as its palatability (Hertwig, 1931). The heated germ remained fresh even after 25 days of storage at 50 ºC in a glass jar. The toasting of wheat germ was reported to be suitable for storing it without affecting its nutritive value (Hove and Harrel, 1943). Protein of roasted wheat germs at 130-150ºC has higher digestibility and protein efficiency ratio than the raw wheat germs which proves that roasting destroyed digestion enzymes inhibitors (Jurkovic et al., 1993). Besides amylolytic and proteolytic activities

decreases by heat treatment at 240ºC for 2 minutes (Wierzbowski et al., 1966). Rao et al. (1980) found that toasting of wheat germ at 150 ºC in an air circulation oven for 25 minutes, at 130 ºC in a coffee roaster or for 5 minutes, resulted in a product of improved acceptability and having a residual lipase activity of 76.4 and 45.9% respectively.

Infrared heating increased the stability of germ (Maes and Bauwen, 1951). The stability was dependent on the thickness of the germ layer, the intensity and the period of the heating.

The microwave treatment on rice bran, wheat germ and soybean were analyzed by Vetrimani et al. (1992). Results showed that microwave treatment led to considerable inactivation of lipase and complete inactivation of lipoxygenase present in these materials.

The effect of heating germ at 100ºC, various moisture levels (1-11%) for different periods (1.5 hr, 12 hr) in a hot air oven, on the enzyme activities and its stability during storage at 37 ºC in a laminated metal foil was studied by Lusena and Mc Farlane (1945). They underlined that all the heat treatments completely destroyed lipoxidase activity and considerably the proteolytic activities were reduced, depending on the moisture level. Lusena and Mc Farlane (1945) treated raw germ with moist heat followed by dry heat since the germ with low moisture content showed fewer tendencies to develop off-flavors in storage. The treated samples packed in cellophane envelopes were in good condition even after a month's storage at 55 ºC.

Rothe (1963) has reported that the 100% lipase activity observed in germ samples containing 26.5 % moisture decreased steadily to complete inactivation, when the moisture content was reduced to about 4% by a simple drying at 80-100ºC.
Studies on the heat stabilization of wheat germ by using hot air-steam at 80º-110ºC or steam heating at 0.5-1.75 atmospheres followed by hot air stream drying

at 100º-120ºC (Ivanova et al., 1975). Drying in a hot air stream at 100ºC was also found to be most promising treatment. Heating at 100ºC for 8 min. reduced moisture from 12.67 to 2.90% and germ could be stored for 90 days without any change in its vitamin E content.

Rao et al. (1980) concluded that steaming of wheat germ at atmospheric pressure totally inactivated lipase, lipoxidase and proteolytic enzymes in the product. Drum drying of slurry containing 33% ground raw germ at a steam pressure of 35psi and at a drum temperature of 138ºC resulted in highly acceptable product, low in enzyme activity. In that study unprocessed germ stored in polyethylene pouches turned unacceptable within 2 weeks of storage while defatting of germ improved shelf life to 12 weeks. Drum drying extended the shelf life to 20 weeks, whereas toasting or steaming increased it to 26 weeks.

Wheat germ can be fluidized by hot air due to its low density and non-sticky surface characteristics. A spouted bed is a kind of fluidized bed, consisting of a cylindrical vessel with conical bottom fitted with only one inlet nozzle for the gas injection (Mujumdar and Passos, 1987). Spouted bed dryer has some advantages as uniformity in bed temperature and moisture content of product, high inlet gas temperature without thermal damage of product, lower capital and operating costs and elimination of localized overheating of product. By using a spouted bed good circulation can be obtained by less fan power. Yöndem-Makascıoğlu et al. (2005), studied stabilization of wheat germ by roasting in a spouted bed with air at 140º- 200 ºC for 3-9 minutes. The lipase activity was decreased by 6-65 %. Wheat germ processed at 200ºC for 6 minutes ranked highest in sensory evaluation, described as having ‘a golden color’ and ‘nutty flavor’, and its lipoxygenase activity had decreased by 91.2 %.

A spouted bed unit for roasting and cooling of wheat germ were constructed by Oymak (2006) through his thesis. The drying temperatures ranged between 201º

and 243ºC, operation times between 7 and 12 minutes, and air flow rate between 55 and 65 m^3/ h. It was seen that the degree of roasting was closely related to exit temperature. The optimum exit air temperature range was determined as 155º-160ºC. The germs processed at 209ºC for 12 min. with 60 m^3/h air flow rate and at 216ºC for 7 min with 55 m^3/h flow rate were selected as the sample for storage studies according to the results as sensory evaluation tests. Processed germs at stated conditions had shelf lifes increased by 8-10 fold when compared to raw samples.

It has also been reported (Krings et al., 2006) that roasting of wheat germ introduces DNA protective properties to its ethanolic extracts, not observed in the extracts of raw germ.

1.1.4.3 STORING CONDITIONS FOR WHEAT GERM

Sherwood et al. (1933) studied the shelf life of whole wheat germ packed in vacuum or with inert gases like nitrogen or carbon dioxide at various temperatures. In this study alcoholic acidity was used as a measure of the freshness or the spoilage of the germ during storage. Considering acidity changes as well as organoleptic quality, vacuum-packed germ was found to be better than that packed under inert atmosphere, and the shelf life of wheat germ stored under vacuum between -10º-7ºC was minimum 6 months.

Yakovenko (1961) observed an increased lipoxidase activity in stored wheat germ which was dependent on the temperature of storage. Maximum lipoxidase activity was observed during 6 months of storage at 16º-21ºC.

After all Yöndem- Makascıoğlu et al. (2005) compared the processed (spouted bed drying, 200ºC for 6min.) and raw wheat germ stored in paper, polyethylene

and vacuum-packed polyethylene pouches at 5º-40 ºC. The shelf life increased in the order paper, polyethylene, vacuum-packed polyethylene.

1.2 ANTIOXIDANTS

A free radical can be defined as any atom or molecule that posses an unpaired electron. It can be anionic, cationic or neutral. In biological and related fields, the major free radical species of interest have been those of oxygen, called oxygen free radicals (OFRs) (Punchard and Kelly, 1996).

OFRs are part of a greater group of molecules called reactive oxygen species that are all more strongly oxidizing than molecular oxygen itself .This group of molecules includes hydrogen peroxide (H_2O_2), lipid peroxide (LOOH),singlet oxygen (1 O_2), hypochlorus acid (HOCl) and other n-chloramine compounds. OFRs are not the only free radical species. Carbonyl, thiyl and nitroxyl radicals are also important radical species. Many other free radical species can be formed by biological reactions. OFRs are potentially very toxic to cells. Due to their high reactive nature they can readily combine with other molecules within the cell such as enzymes, receptors and ion pumps nature, causing oxidation directly, and inactivating or inhibiting normal function of cells (Punchard and Kelly, 1996).

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

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. Such kind of chain reactions should be terminated before giving substantial damages on different parts of the cell. Any food will eventually oxidize even if it is stored at temperatures as low as - 70°C (Punchard and Kelly, 1996). Therefore, degradation of food occurs, and unpleasant taste and odor develops.

The term food antioxidant is generally applied to those compounds that interrupt the free-radical chain reactions involved in lipid oxidation. Antioxidants are classified into five groups (Rossel and Kochhar, 1990) as follows:

i. 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. 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-) 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 such as alkyl gallates, butylated hydroxyanisole (BHA), butylated hydroxytoluene (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).

ii. Oxygen Scavengers;

Ascorbic acid (Vitamin C), ascorbyl palmite, erthorbic acid (d-isomer of ascorbic

acid) and its sodium salt etc. belong to this group of antioxidants. They can react with oxygen and can thus remove it in a closed system.

iii. 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 hydro peroxides to non-radical species, absorb UV radiation or deactivate singlet oxygen. Dilauryl thiopropionate and thiodipropionic acid are examples of this category.

iv. Enzymic Antioxidants;

These groups 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.

v. Chelating Agents ;

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.

1.2.1 PHENOLIC COMPOUNDS

In organic chemistry, phenols, sometimes called phenolics, are a class of chemical compounds consisting of a hydroxyl group (-OH) attached to an

aromatic hydrocarbon group. The simplest of the class is phenol (C_6H_5OH). Although similar to alcohols, phenols have unique properties and are not classified as alcohols (since the hydroxyl group is not bonded to a saturated carbon atom). They have relatively higher acidities due to the aromatic ring's tight coupling with the oxygen and a relatively loose bond between the oxygen and hydrogen. Polyphenols are a group of chemical substances found in plants, characterized by the presence of more than one phenol group per molecule. Polyphenols are generally further subdivided into hydrolysable tannins, which are gallic acid esters of glucose and other sugars; and phenylpropanoids, such as lignins, flavonoids, and condensed tannins. They are primary antioxidants and singlet oxygen quenchers (Kandawasmi et al., 1994). They are also very powerful metal chelating agents. They can trap free radicals and break chain initiation reactions. Flavonoids have structural variations in carbon ring that identifies the different types, namely, flavanols, flavones, isoflavones, flavonones, flavonol and anthocyanins (Shi et al., 2003).

Polyphenols are reducing agents, and together with other dietary reducing agents, such as vitamin C, vitamin E and carotenoids, they protect the body tissues against oxidative stress. Commonly referred to as antioxidants, they may prevent various diseases associated with oxidative stress, such as cancers, cardiovascular diseases, inflammation and others (Scalbert and Williamson 2000).

1.2.2 TOCOPHEROLS (VITAMIN E)

Vitamin E emerged as an essential, fat soluble nutrient that functions as an antioxidant in the human body. It must be provided naturally by foods or by additive intakes.

Vitamin E represents an antioxidant family comprised of tocopherols and

tocotrienols. In nature four different forms (α-, β-, δ-, γ-) of tocopherols and tocotrienols occur (Figure 1.2). The α- form of tocopherols and tocotrienols has three methyl groups, the β- and γ- forms have two methyl groups, and the δ- form has one methyl group on the aromatic ring (Sen et al., 2006).

Vitamin E content or the activity is usually represented by the amount of α-tocopherols. As such, α-tocopherol is considered to have the highest nutritional value of the different forms of tocopherols and tocotrienols, and has thus received the greatest attention for the vitamin E biofortification of crop plants. By comparison, α -tocotrienol has about one third of the vitamin E activity displayed by α-tocopherol, while γ-tocopherol has only about one tenth of the vitamin E activity of α–tocopherol (Kamal-Aldin et al., 1996).

What makes α -tocopherol such a highly efficient antioxidant is (i) that it reacts with the peroxyl radical extremely fast, much faster than to allow for the peroxyl radical to do any other reactions; (ii) it takes away the radical character from the oxidizing fatty acid and prevents it from further radical reactions; (iii) in the antioxidant reaction, α-tocopherol is turned into a fairly stable radical. Under normal circumstances, it will only react with another radical (either a tocopheroxyl radical or a fatty acid peroxyl radical) to form stable, nonradical products (Schneider, 2005). α -tocopherol is practically insoluble in aqueous solutions, but freely soluble in oils, acetone, ethanol, ether, and other organic solvents.

Of the different forms of tocopherol, δ- and γ- tocopherols are the most effective at reducing the oxidative breakdown of vegetable oils in frying applications. In addition, δ- and γ-tocotrienols have a slightly greater ability than the corresponding tocopherols to reduce the formation of fatty acid oxidation (Hunter and Cahoon, 2007)

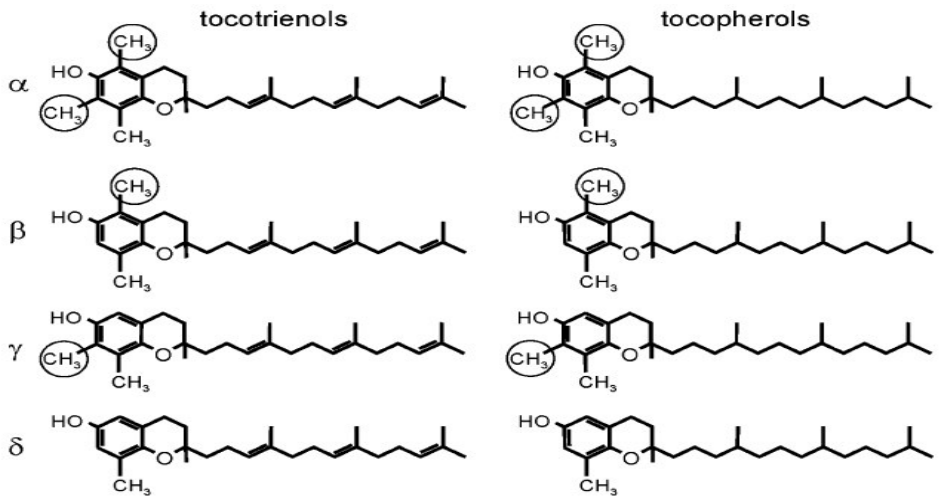

Figure 1.2 Tocopherols and Tocotrienols (Hunter and Cahoon, 2007)

In order to prevent oxidation reactions and to extend the shelf life of products, 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 BHA and BHT are powerful inhibitors for lipid peroxidation. However, long-term in vivo studies have been reported that they could promote tumor formation (Barlow, 1990). Regarding long term health concerns, in food processing industry a demand for natural ingredients with antioxidant activity arose. As a consequence extraction of antioxidants from food sources became a promising research area.

1.2.3 ANTIOXIDANTS IN WHEAT GERM

Wheat and wheat-based food products are shown to have antioxidant activities (Yu et al., 2002b). Anderson and Perkin (1931) characterized a water insoluble flavone, tricin, from wheat. As compared to control $DPPH^{\cdot}$ solution (100μM), wheat extracts showed greater radical $DPPH^{\cdot}$ scavenging activity than vitamin E,

vitamin C, and the well known synthetic antioxidants each having 50μM concentration (Yu et al., 2002b). Not only wheat but wheat germ is also reported to demonstrate strong antioxidative activity. In 1936 it was discovered that vitamin E was abundant in wheat germ oil and two years later it was chemically synthesized for the first time (Sen et al., 2006).

In wheat germ, King et al. (1962) found glycoflavones, ferulic acid and vanillic acid in the free form. Total phenolic content (TPC) was reported to be 3.49 mg / g wheat germ as ferulic acid equivalents.

In addition to tocopherols and phenolics, carotenoids were characterized from wheat germ oil by SC-CO_2 extraction (Panfili et al., 2003). The most abundant carotenoid in wheat germ oil was lutein (4.77 mg/100 g germ oil), followed by zeaxanthin (3.73 mg/100 g germ oil) and β-carotene (1.10 mg/100 g germ oil).

1.3 EXTRACTION OF ANTIOXIDANTS

As the new trend of "natural additives" arose, extraction methods of antioxidants became more important. An ideal extraction procedure should be rapid, simple and inexpensive.

1.3.1 TRADITIONAL SOLVENT EXTRACTION

The traditional solvent extraction (TSE) requires long extraction times, large volumes of solvents and disposal of toxic solvents. Moreover, it causes the degradation of thermally liable natural products at high temperatures.
Aqueous alcohols and acetone, with different levels of water, have been widely

used to extract phenolic components from botanical materials, especially herbs. Normal hexane is commonly used for edible oil extraction. Ethanol, isopropanol, acetone and isohexane are proposed as alternative organic solvents to hexane (Dunford et al., 2003).

Extracts of roasted wheat germ (160ºC, 20 min) obtained using different solvents were compared by Krings et al. (2000) and all retarded the autoxidation of corn oil stored at 60ºC. Acetone extracts of roasted wheat germ provided a slight stabilization of genuine corn oil, but beneficial effects are recorded with diethyl ether and ethanol extracts. The increases of peroxide value and conjugated diene concentration were delayed; so was the decomposition of α-tocopherol.

Further, the antioxidative potential of ethanolic extracts from roasted wheat germ was compared with extracts from other roasted food. Among the cereal products, roasted wheat germ and roasted press cake from wheat germ processing yielded the most efficient extracts when added to stripped maize oil under accelerated-oxidation conditions. $DPPH^{\cdot}$ scavenging activity of the hazelnut extract was significantly higher than wheat germ and close to coffee, while the sweet almond extract showed poor radical-scavenging. Total phenols in ethanolic extract of roasted wheat germ are reported as 22 μg mL^{-1}(Krings et al., 2001). Also, roasted wheat germ extracts demonstrated higher DNA protective activity than coffee extract (Krings et al., 2006).

Liyana-Pathirana et al. (2006) investigated the 80% ethanolic extracts of soft and hard winter wheat samples and their milling fractions, namely flour, germ, bran and shorts. The authors concluded that wheat germ exhibited the highest total AA, followed by the shorts, bran, whole grain and flour for both soft and hard wheat. The germ fraction also possessed the highest TPC. The germ and flour fractions demonstrated the highest and lowest $DPPH^{\cdot}$ radical scavenging activity, respectively, among wheat fractions.

Significant difference in total TPC was observed among the various solvent extracts of wheat bran in the work of Wang et al. (2008). Ethanol extracts contained highest TPC, followed by methanol and acetone extracts. Also using aqueous ethanol as solvent proved the extractions of phenolics compared to pure ethanol. Optimum results were obtained when ethanol content was about 60% in solvent.

1.3.2 MODERN SOLVENT EXTRACTION TECHNIQUES

Alternative extraction techniques that reduce the volume of solvent and the time of extraction have been developed. For example, some of the new techniques are pressurized liquid extraction, microwave extraction, ultrasound assisted extraction (UAE) and supercritical fluid extraction.

1.3.2.1 ACCELERATED SOLVENT EXTRACTION

Accelerated solvent extraction is also referred as pressurized solvent extraction (PSE). Pressurized liquid extraction is performed at high pressures and temperature above the normal boiling point of the organic solvent. The use of higher temperature increases the ability of solvent to solubilize the analyte, decreases the viscosity of liquid solvents, allowing better penetration of the solvent into the matrix. The use of higher pressure facilitates the extraction of the analytes from samples by improving the solvent accessibility to the analytes that are trapped in the matrix pores (Peres et al., 2005).

Dunford et al. (2003) examined the PSE of wheat germ oil. The extracts were analyzed for n-3 and n-6 polyunsaturated fatty acid content. PSE reduced extraction time significantly as compared to Soxhlet extraction and had no

adverse effect on the fatty acid composition of the oil. The effects of temperature (43º-45ºC at 103 psi), extraction time, sample size and solvent type on the extraction efficiency and oil quality were studied. Extraction efficiency of the n-hexane was compared to that of the isohexane and high purity hexane, isopropanol, ethanol and acetone. In the study of Dunford et al. (2003), it is pointed out that the amount of collected wheat germ oil was highest when ethanol was used as a solvent for pressurized solvent extraction. The yield in ethanol extracts was two times higher than that in the hexane extracts.

Hasbay-Adil (2006) compared pressurized liquid extraction (with ethanol) with solvent (with methanol and ethanol) and SC-CO_2 extractions. SC-CO_2 extraction was not efficient as solvent and pressurized liquid extraction. TPC (2.08 mg GAE/g sample) of apple pomace extracts obtained by pressurized liquid extraction (1410-1590 bar, 60ºC, 29-36 min.) was higher than those obtained by solvent and SC-CO_2 extraction.

1.3.2.2 MICROWAVE ASSISTED SOLVENT EXTRACTION

The microwave-assisted solvent (MAS) extraction provides successful results because it more evenly heats the sample and allows for higher extraction temperatures and pressures. MAS extraction has been widely used for extractions in order to replace other extraction methods, specifically, traditional solvent extraction method.

This extraction method has numerous advantages over the TSE method. For example, it reduces extraction time or enhances the efficiency of the extraction. Moreover, since it is not tedious and not restricted in solvent selectivity, no concentration and evaporation step is required, and the possibility of contaminating the sample with solvent impurities is much lower (Csiktusnadi

Kiss et al. 2000).

Two types of instruments are commercially available for microwave assisted solvent extraction (Kaufmann and Christen, 2002); Closed vessel (under controlled pressure), Open vessel (microwave-assisted Soxhlet extraction, under atmospheric pressure).

Holliday (2006) compared the AA and TPC of the extracts obtained by TSE (methanol, 25 min. and 60ºC), MAS (10 g oat bran/ 40 mL methanol, 20 min., 60ºC & 100ºC, 0.41 bar, 8000W) and SC-CO_2 (65 bar, 25º-75ºC) extraction from oat bran. Extracts obtained by MAS extraction at 60ºC had higher AA and TPC than those obtained by TSE. Extracts of SC-CO_2 extraction had the highest AA and TPC.

1.3.2.3 ULTRASOUND ASSISTED EXTRACTION

Ultrasound is acoustic (sound) energy in the form of waves having a frequency above the human hearing range. The highest frequency that the human ear can detect is approximately 20 thousand cycles per second (20 kHz). This is where the sonic range ends, and where the ultrasonic range begins. Ultrasound is used in electronic, navigational, industrial, and security applications. It is also used in medicine to view internal organs of the body (Luque Garcia and Luque de Castro, 2003).

The enhancement of extraction efficiency of organic compounds by ultrasound is attributed to the phenomenon of cavitation produced in the solvent by the passage of an ultrasonic wave. Cavitation bubbles are produced and compressed during the application of ultrasound. The increase in the pressure and temperature caused by the compression leads to the collapse of the bubble. With the collapse of

bubble, a resultant ‘‘shock wave’’ passes through the solvent enhancing the mixing. Ultrasound also exerts a mechanical effect, allowing greater penetration of solvent into the sample conmatrix, increasing the contact surface area between solid and liquid phase. This coupled with the enhanced mass transfer and significant cell disruption of cells via cavitation bubble collapse, increases the release of intracellular components into the bulk medium. The use of higher temperatures in UAE can increase the efficiency of the extraction process due to the increase in the number of cavitation bubbles formed (Rostagno et al.,2003).

The application of ultrasound waves have also been reported (Suslick, 1998) to result in the formation of free radicals as a result of very short term local temperature and pressure increases, which may partially consume the antioxidants released from the matrix. Therefore, the optimum duration of ultrasonication may be anywhere between very short and very long times.

UAE of various analytes from a variety of organic and inorganic samples using different types of solvents have been reported in the literature. Ultrasonic bath and ultrasonic probe systems are the two most common devices used in ultrasound-assisted extraction. The UAE is carried out in three ways; indirect or direct sonication using an ultrasonic bath and direct sonication using an ultrasonic probe (Vinatoru et al., 1997). Direct sonication is more effective on extracting solvent and solid. During indirect sonication, effects of ultrasound waves may be buffered due to the presence of a layer around material. Longer sonication times can be needed to get same extraction efficiencies as those obtained by indirect sonication.

Wu et al. (2001) compared direct and indirect ultrasonication with Soxhlet extraction of ginseng saponins from ginseng roots and cultured ginseng cells. It was found that UAE was about three times faster than the traditional extraction method and direct sonication by the probe could provide much higher ultrasound

energy to the samples than indirect sonication by the cleaning bath.

Although ultrasonic baths are more widely used devices, they have two main drawbacks that considerably decrease experimental repeatability and reproducibility (Luque-Garcia and 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.

Rostagno et al. (2003) compared the efficiencies of mix-stirring extraction and ultrasound-assisted extraction, using different solvents (methanol, ethanol and acetonitrile at 30-70%) and extraction temperatures (10ºC&60ºC) in extracting four isoflavone derivatives (daidzin, glycitin, genistin and malonyl genistin) from freeze–dried ground soybeans. The efficiency of the extraction of soy isoflavones was improved by ultrasound but was dependent on the solvent employed. By ethanol (50%), methanol (50%) and acetonitrile (40%) highest yields of total isoflavones were obtained, with only small differences between them. In order to improve extraction of isoflavones from soybean, adding a certain amount of water (40-60%) to the extracting solvent was found to be necessary.

Özcan (2006), studied ultrasound assisted extraction of phenolic compounds from Merlot grape pomace. 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 analyzed by response surface methodology. Highest phenolic content in the extracts (47.2 mg GAE/g powder) was obtained using 30 % aqueous ethanol as solvent, applying 6 minutes of sonication 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 ethanol contents of the solvent with shorter sonication and water bath

times could give higher results.

Usaquen-Castro et al. (2006) pre-treated red grape (*Vitis vinifera*) residues which contain large amounts of polyphenols (especially anthocyanins), with ultrasound at 35 kHz for 15-90 min (240 W). Right after the pre-treatment, the samples were subjected to an extraction operation by methanol/water (96% v/v) at 60°C over 90min. Both total extractive yield (dry basis) and TPC were determined for all the extracts obtained from the treated samples, and compared against an extract obtained from a non treated sample. The effect of ultrasound on the AA of the extracts was also evaluated. Authors showed that at the studied frequency (35 kHz), there is a significant effect ($p \leq 0.05$) of the ultrasound exposure time on both total extractive yield and TPC. When compared to non treated samples, the exposure to ultrasound at 45 min. improves the extraction by increasing in more than 25% the total extractive yield (except at 75min), also ultrasound at 45, 75 and 90min increased the TPC of the extracts in about 65, 50 and 140%, respectively. However ultrasound at 35 kHz decreases the AA of the extracts about 50%.

Melikoğlu (2005) aimed to extract lipids and antioxidants from wheat germ using an ultrasonic bath. Germ to solvent ratios of 1/1, 1/2 and 1/5 proved to be inadequate. Using 1/10 and smaller ratios, well mixing was observed. Ethanol, isopropanol and acetone were compared for extraction purposes. Ethanol presented higher extraction yields than isopropanol and acetone. Ethanol extracts from both roasted and non roasted wheat germs were characterized in terms of their TPC. TPC of non-roasted and roasted wheat germ obtained by 30 minutes of UAE were 2 mg GAE/ g germ and 1.7 mg GAE/ g germ, respectively.

Ultrasonication time was reported to be the most significant parameter for the extraction of phenolic compounds from wheat bran using UAE (40 kHz, 250W) (Wang et al., 2008). Ethanol level (20-95%), temperature (25º-75ºC) and time

(10-50 min) were studied as parameters of UAE. The optimum extraction conditions were found to be 64% ethanol, 60ºC and 25 min. Under those conditions, the TPC of the extracts was 3.12 mg GAE/g wheat bran.

1.3.2.4 SUPERCRITICAL FLUID EXTRACTION

The critical temperature, Tc, of a material is the temperature above which distinct liquid and gas phases do not exist. The critical pressure, Pc, is the vapor pressure at the critical temperature. As the critical temperature is approached, the properties of the gas and liquid phases become the same resulting in only one phase: "the supercritical fluid" (Sihvonen et al., 1999). Supercritical fluid extraction has become an alternative to the conventional extraction techniques. This is mainly because dissolving power of supercritical fluids can be adjusted by simply changing the pressure and temperature and there is no need for solvent separation after the extraction. It is more environmentally friendly than the classical methods. The initial high investment cost involved in supercritical fluid technology can therefore be paid off through the benefits gained for the end products.

Some disadvantages of supercritical fluid extraction compared to conventional liquid solvents for separations are; required elevated pressure, compression of solvent requires elaborate recycling measures to reduce energy costs and high capital investment for equipment. Supercritical carbon dioxide (SC-CO_2) has been the most commonly used solvent in the food and pharmaceutical industries, since it is non-toxic, non-flammable, chemically stable, inexpensive, environmentally acceptable and easily separated from the extract.

As a supercritical fluid, carbon dioxide (CO_2) has unique physicochemical properties, such as high density and low viscosity that make it suitable as an

extraction solvent. Also considering its moderate T_C (31.1 ºC) and P_C (73.8 atm), availability, nonflammability, and low cost carbon dioxide is utilized (Reverchon 1997).

The use of CO_2 as a supercritical solvent permits the processing of thermo sensitive material (Reverchon et al. 1993). However, SC-CO_2 has been shown to be a poor solvent for polar compounds. An alternative solution is cosolvent-modified SC-CO_2. Addition of organic co-solvents like ethanol, methanol, acetone, increases the solvating power of CO_2 and the yield of extraction of polar solvents such as polyphenols. Ethanol is a permitted co-solvent in food industry. Consumption of ethanol is not in large amounts as in conventional solvent extraction, and it is easily eliminated from the extract by evaporation at room temperature.

Quality of wheat germ oil extracted by liquid and SC-CO_2 was explored by Molero Gomez et al. (2000). The extraction of wheat germ oil by liquid and SC-CO_2 was described from the point of view of both operative method and pretreatment of raw material. The optimization of the extraction conditions were performed at between 50 and 300 bar; 10º and 60°C; 0.5 and 2.0 L/min CO_2 flow rate at standard temperature and pressure; for 3 hours. The extraction vessel was 75 mL in volume and 25 g germ was placed in extractor for experiments. The best conditions for wheat germ oil extraction were found to be: 150 bar, 40ºC at 1.5 L/min CO_2 flow rate. The yield (8.0 % wheat germ oil/ wheat germ) and fatty acid compositions of the extracts were very similar to those of Soxhlet extraction by hexane (8.6 % wheat germ oil/ wheat germ) for 16 hour, although a higher-quality oil was obtained by SC-CO_2 extraction (416.7 mg tocopherol/ g wheat germ oil - 33 mg tocopherol/ g wheat germ).

Panfili et al. (2003) studied the condition for the extraction of wheat germ oil in a SC-CO_2 pilot plant of 1 liter extraction capacity using 600–700 g of milled wheat

germ placed in the extraction vessel. The CO_2 flow rate was 1.5 L/min. The experimental conditions adopted during extraction were as follows: (i) P = 250 bar, T = 55°C, wheat germ particle size = 0.50 mm; (ii) P = 380 bar, T = 55°C, wheat germ particle size = 0.50 mm; (iii) P = 380 bar, T = 55°C, wheat germ particle size = 0.35 mm. Particle size appears to have a greater influence on yield than the pressure adopted during extraction. The best conditions were found to be 380 bar, 55°C and wheat germ particle size about 0.35 mm. These conditions gave yields of about 92% of total oil (11.7 g oil/100 g wheat germ) after 3 h of processing. The obtained oils and the partially defatted cake were investigated with regard to their fatty acids, tocopherol and tocotrienol, carotenoids, and sterol compositions and to their quality characteristics. Thus, reduction of the particle size increases diffusivity to a critical point. Tocopherol extracted at best conditions for 75 min yielded 2.926 mg tocopherol/g wheat germ oil (0.342 mg tocopherol/g wheat germ).

Natural vitamin E was extracted by SC-CO_2 from wheat germ by Ge et al. (2002a). They developed an efficient supercritical fluid extraction process with carbon dioxide for the extraction of natural vitamin E from wheat germ and to ensure maximal vitamin E yield optimized both the pretreatment of extracted wheat germ and extraction conditions. Wheat germ (up to 5.0 g) was packed in a sample cartridge with the volume of 10 mL. Vitamin E yield increased with reduced water content to 5.1 %; however when the water content was reduced to 4.3% vitamin E yield also decreased. Besides particles sizes smaller or larger than 0.505 mm gave lower extraction yields. The extraction was undertaken at 276 to 345 bar, 40 to 45ºC, the carbon dioxide flow rate of 1-3 mL/min for 90 min. Maximal vitamin E yield (18.38 mg tocopherol/g) was recorded for a flow rate of 2mL/min, 90 min extraction time at 207 bar with a particle size of 0.505 mm and 5.1% moisture content. It was observed in this study that reduced water content (<5.1%) resulted in the shrinking of germ particles. They suggested that vitamin E transfer was therefore more difficult. Increased temperature enhanced the

diffusion coefficient of the extracted molecule but reduced the density of the CO_2 and therefore the saturated solubility of the extract. Total amount of tocopherol extracted at 276 bar, 40ºC and 2 mL/min flow rate for 90 min yielded 21.79 mg tocopherol/g.

Further Ge et al (2002b) optimized the extraction of natural vitamin E from wheat germ using response surface methodology. The extractor vessel was a 10 mL volume and a sample of about 5 g was placed in the extractor vessel. Extracting pressure, temperature, and flow rate of carbon dioxide were examined as the independent variables of central composite rotate design. Through the response surface methodology, the optimal processing conditions were determined. Pressure (138-413 bar), temperature (30º-50ºC) and flow rate (1.5-2.5 mL/min) were optimized. Their results demonstrated that pressure, temperature, pressure × temperature interaction, and flow rate of CO_2 significantly affected the yield of the natural vitamin E, while two interactions containing the flow rate of CO_2 had no significant effect on the yield of natural vitamin E. The optimal processing conditions were found to be: extracting pressure 345 bar, extracting temperature 43ºC, and flow rate of carbon dioxide 1.7 mL/min. They predicted optimum value for the yield of natural vitamin E by response surface methodology as 23.07 mg/g.

Other than the studies summarized above, Eisenmenger et al., (2006) focused on pilot-scale SC-CO_2 extraction and fractionation of wheat germ oil. In that study SC-CO_2 extraction and fractionation techniques were examined as alternative methods to obtain wheat germ oil of high quality and purity. The pilot-scale SC-CO_2 extraction of wheat germ was carried out with three vessels (4 L each) in parallel and each vessel contained 1.5 kg of wheat germ. The total of 4.5 kg of wheat germ was extracted at 80°C and 690 bar using 16 kg CO_2 (measured at atmospheric conditions). The CO_2 flow rate was 0.18 kg/min. The extract was collected in a receiver maintained at 60°C and 110 bar. The highest extract yield

was obtained at the highest temperature and pressure examined in the study (80°C and 550 bar). It was shown that the SC-CO_2 extraction technique is effective in extraction of wheat germ oil. There was no significant difference in the fatty acid composition of SC-CO_2 and hexane extracts. Both were rich in α-tocopherol. Moisture content of the SC-CO_2 extracted oil was higher than that of the hexane-extracted oil. Solvent/feed ratio had a significant effect on the supercritical carbon dioxide extraction yields. Their study demonstrated that supercritical fluid fractionation was a viable process to remove free fatty acid efficiently from both hexane and SC-CO_2 wheat germ oil while retaining bioactive oil components in the final product. 26.86 mg tocopherol/ g oil was extracted by SC-CO_2 extraction technique

Bruni et al. (2002) compared the UAE and supercritical fluid extraction for the extraction of Vitamin E isomers from *Amaranthus caudatus* seeds. Three different methods; solvent extraction with methanol (at constant 1/20 solid/solvent ratio, 25ºC for 24 hour and dried by rotatory evaporator), indirect sonication (5 g/100 mL methanol 25ºC for 1 hour and centrifuged) and SC-CO_2 extraction (CO_2 flow rate 2L/min, 40ºC, restrictor temperature: 70ºC at 202.65 bar and 405 bar 15 min extraction). Solvent extraction yielded 0.076 mg tocopherol/ g seed, Ultrasonication yielded 0.064 mg tocopherol/g seed and SC-CO_2 yielded 0.1 mg tocopherol/ g seed (at 203 bar) and 0.13 mg tocopherol/ kg seed (at 405 bar). Supercritical fluid extraction gave quantitatively better yields of tocopherols in shorter times with solvent free extracts. Ultrasound extraction gave qualitatively acceptable results more rapidly than the classical methods.

1.4 RESPONSE SURFACE METHODOLOGY

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).

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. 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.

On the other hand, polynomial models have some disadvantages like; impossible extrapolation outside of the range, being symmetrical about the optimum and not including a form that can asymptotically approach a constant response level. 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 (Myers and Montgomery, 1995).

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).

1.4.1 SECOND ORDER CENTRAL COMPOSITE DESIGN

Central composite designs include three types of experimental points; factorial points, star points and replicated points (Thompson, 1982). Following sections explain those points.

Factorial 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. If number of explanatory variables or independent variables is represented by k, the number of factorial points is;

$$n_c := 2^k \tag{1}$$

Star points have coordinates of (± α, 0, …, 0). 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;

$$\alpha := n_c^{\frac{1}{4}} \tag{2}$$

The number of star points (n_α) is;

$$n_\alpha := 2^k \tag{3}$$

Replicated points 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 is found by the following equation;

$$n_o := \frac{4 \cdot \alpha^2 \cdot \left(n_c + \alpha^2 \right)}{n_c} - n_\alpha \qquad (4)$$

1.5 OBJECTIVE OF THE STUDY

Several studies are reported about extraction of antioxidants from roasted wheat germ using UAE and SC-CO_2. In literature it is clearly shown that ethanol efficiently extracts phenolics from roasted wheat germ; however, ethanol-water mixture, being widely reported in literature to be a good solvent for phenolics, have not been tried on wheat germ. Moreover, short time extraction was not looked into. Although tocopherol contents were reported, phenolic content and antioxidant activities of SC-CO_2 extracted wheat germ oil have not been specified (Ge et al., 2002a & 2002b, Panfili et al., 2003, Gomez et al., 2000)

The aim of this study was to optimize extraction conditions for direct application of ultrasound in terms of three dependent variables; extraction yield %, total phenolic content, TPC, (mg GAE/ g extract) and antioxidant activity, AA, (mg scavenged $DPPH^{\cdot}$ / g extract) by using three level five factorial central composite rotatable design. Extraction parameters were ultrasonication time, Ethanol level (%) and extraction temperature (ºC). Addition to ultrasound assisted extraction; optimization of SC-CO_2 extraction for same dependent variables was aimed.

Differing from literature, a wide range for P was used. Pressure (bar), temperature (ºC) and extraction time (min) are dependent responses. Besides TPC, AA and total tocopherol content (TTC) of the extracts were determined for both extraction methods.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

The raw wheat germ used in this study was obtained from Ankara Un Sanayii A.Ş. In this research germs were immediately stabilized by using a pilot scale conventional spouted air bed dryer at 216 ºC with an air flow 55 m^3/h for 7 minutes. Details of drying are given A.1. After roasting, germ was quickly emptied in a glass jar, closed tightly and cooled to room temperature, finally stored at – 18 ºC in polyethylene pouches.

Before extractions sieve analysis was applied in order to determine the particle size distribution of roasted and milled-roasted wheat germ (Appendix A.1). Also moisture content (AACC Method 44-20), crude fat analysis (AACC Method 30-25) and ash analysis (AOAC Method 923.03) were made. All procedures are presented in appendix A.2-A.5.

Absolute grade ethanol was Delta (99.5 %, Ankara, Turkey) and ultra pure water processed by HUMAN RO 180 and HUMAN UP 900 (Human Corp., Seoul, Korea) was used. $DPPH^{\cdot}$ (2,2-diphenyl-1-picrylhydrazyl radical) (Sigma-Aldrich, Steinheim, Germany). Folin & Ciocalteu's Phenol Reagent, anhydrous sodium carbonate, petroleum benzene and trichloroacetic acid (TCA) (Merck, Darmstadt, Germany) were used in the analyses.

Following Figure 2.1 sets out the whole procedure step by step for each method;

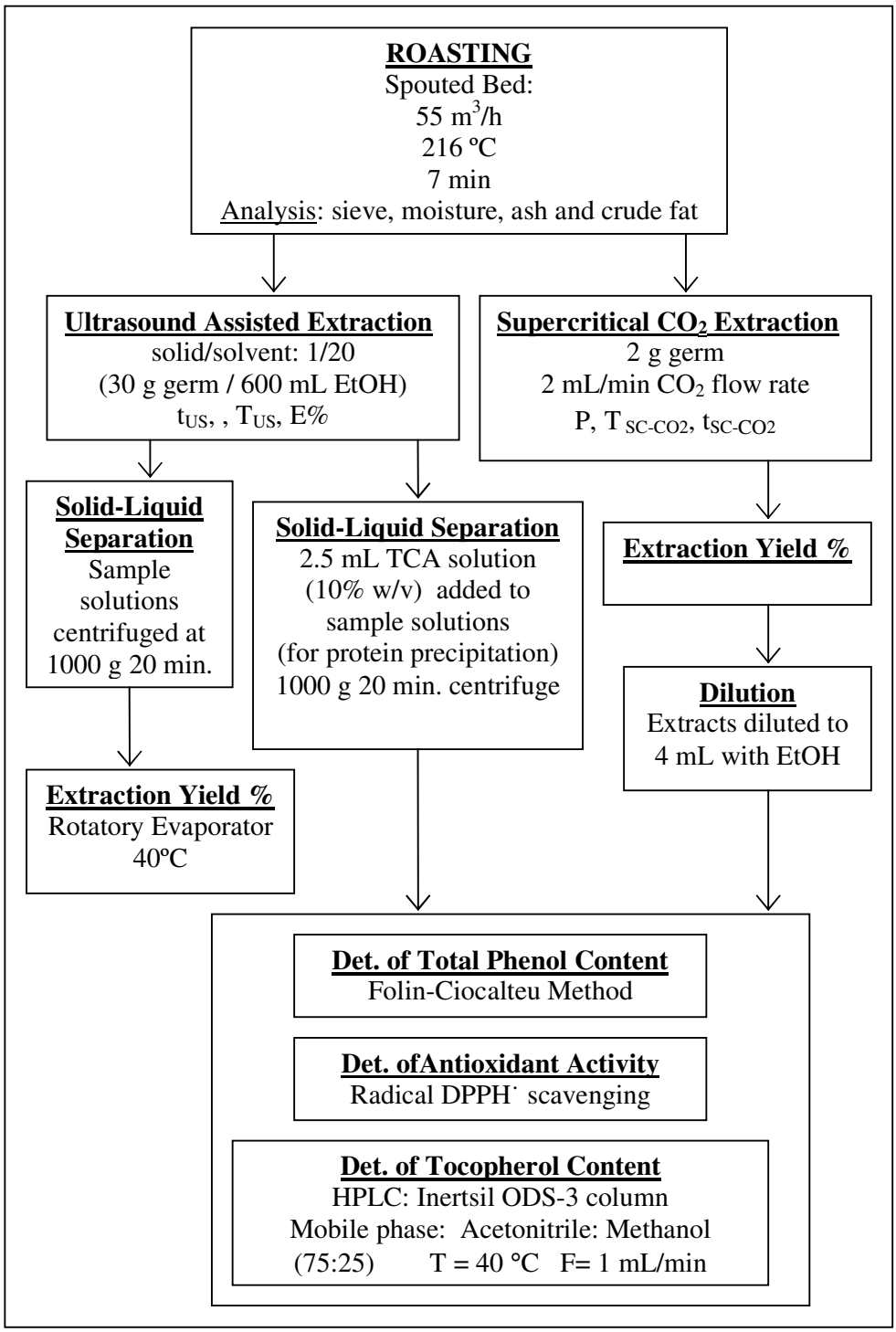

Figure 2.1 Experimental Summary

2.2 ULTRASOUND ASSISTED EXTRACTION (UAE)

Direct UAE performed in an ultrasonic bath "SONOREX SUPER RK 31 H" (Bandelin Electronic, Berlin-Germany). It has an intake of 0.9 L, ultrasonic peak out of 240 W, frequency of 35 kHz, nominal voltage of 230 V~ 50 / 60 Hz, a built-in-heating (heating capacity: 70 W) and time control unit.

The effects of ultrasonication time (1-11 min), temperature (20-60ºC) and ethanol level % (5-95 %) were investigated. Parameter levels and design of experiment are presented in Tables 4.1 and 4.2, respectively. Solid/solvent ratio was kept constant at 1/ 20. 30 g of weighed wheat germ was mixed with 600 mL solvent at a specified temperature for each run, T_{us}. For each data point, extraction solvent was prepared by mixing water and ethanol at specified ethanol percentage, E %. Each run was carried out for sonication time t_{us}. Through sonication temperature was kept constant at T_{us}.

After ultrasonication, before determining extraction yield, centrifugation was used to separate solid particles from liquid phase. Samples were centrifuged (International Centrifuge Model CS, A Damon Company, Needham, Massachusetts) at 1000 g for 20 minutes.

Since phenolics and vitamins can be heat sensitive for extraction yield analysis, 100 mL sample containing ethanol and water mixture was concentrated by the use of a rotatory evaporator operating (BIBBY, model RE 100 (Rotary Evaporator) and model RE 100 B (Water Bath), Bibby Sterlin Ltd, Stone Staffordshire, England) operating at 40ºC. Collecting flask was weighed before and after evaporation ($w_{f.i}$: empty collecting flask was weighed, w_f: after cooling weight of flask was recorded). Amount of extract for run was calculated, E = w_f - $w_{f.i}$. It is known that 30 g roasted wheat germ was extracted with 600ml solvent.

For each run extraction yield is easily calculated, after E values per 100 mL sample is found, $E.Y_u$ % = ((E*6)/30)*100.

2.3 SUPERCRITICAL CO_2 (SC-CO_2) EXTRACTION

Extractions were done by Supercritical Fluid Extraction System (Figure 2.2)(SFX System 5100, ISCO Inc., Lincoln, NE, USA), which consists of an extractor (SFX 3560) and two syringe pumps (Model 100DX) at R&D-Training Center Central Laboratory - METU.

2 g of sample was placed into 10 mL sample cartridge. The CO_2 flow was downward with a rate of 2 g/min. The restrictor temperature was 100 °C. The extract was collected in a test tube to determine yield and then diluted to a constant volume of 4 ml with pure ethanol for further analysis.

Effects of extraction pressure (148-602 bar), extraction temperature (40-60ºC) and extraction time (10-60 min) were studied at the conditions designated by experimental design. Detailed experimental design is explained in 2.5 and presented in Tables 2.3 and 2.4.

Extraction yield of extracts were simply calculated. For each run roasted wheat germ was weighed, w_g, (approximately 2 g) and used. Empty collection tube was weighed, w_{ti}, before extraction. After extraction and cooling to room temperature collected extract and tube weighed, w_{tf}. Amount of extract is $E_{SCO2} = w_{tf} - w_{ti}$. Extraction yield, $E.Y_{SCO2} = (E_{SCO2}/ w_g)*100$, was recorded.

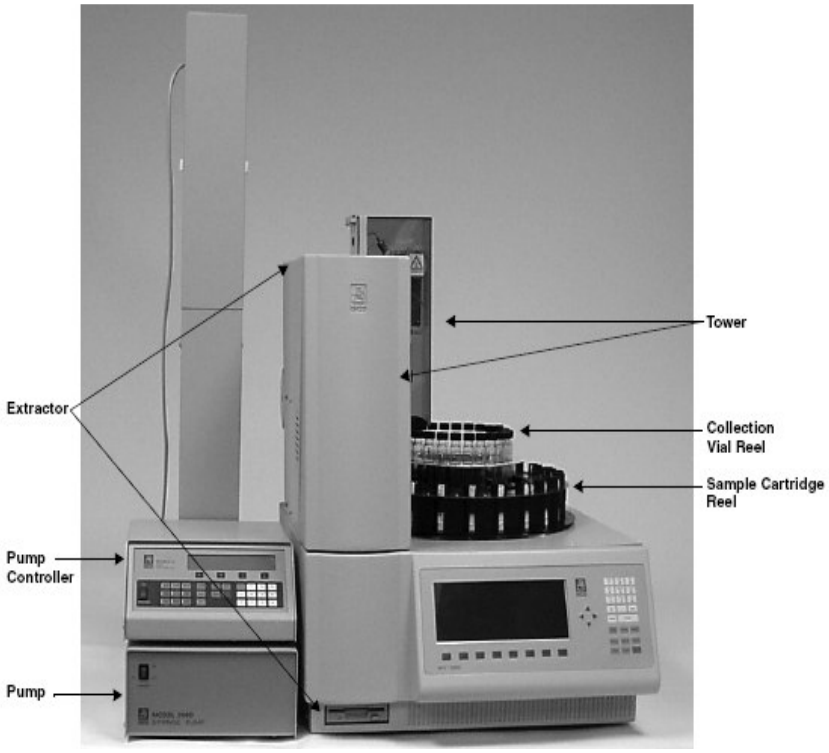

Figure 2.2 SFX 3560 Extraction System

2.4 CHARACTERIZATION OF THE EXTRACTS

After ultrasonication, before analysis, centrifugation was used to separate solid particles from liquid phase were separated in order to determine yield, total phenol content and total antioxidant activity. Since 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, 2.5 mL solution of 10 % (w/v) TCA was added to 50 mL sample analysis tubes before the centrifugation at 1000 g for 20 minutes.

2.4.1 DETERMINATION OF TOTAL PHENOLIC CONTENT (TPC)

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 et al., 1927), which was later improved by Singleton et al. (1965). The Folin-Ciocalteu Reagent is an oxidizing agent consisting of heteropolyphosphotungstate-molybdate (Mo-W). 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 (Activin, 2008).

60 µL samples were diluted with 4.77 mL water, Folin's Reagent of 0.3 mL and then 0.9 mL of 20 % sodium carbonate solution were added to the mixture. The final mixture was kept at 40 °C in shaking water bath for 30 minutes for completing reaction. The absorbance was measured at 766.2 nm by Hitachi-3200 UV-Visible Spectrophotometer (Hitachi Ltd., Tokyo, Japan). Results were reported as gram gallic acid equivalents per gram extract. Details are indicated in Appendix A.6.

2.4.2 DETERMINATION OF ANTIOXIDANT ACTIVITY (AA)

A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical, 2, 2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$). $DPPH^{\cdot}$ is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods. It also has been used to quantify antioxidants in complex biological systems. It is not specific to any solid or liquid samples and to any particular

antioxidant component, but applies to the overall antioxidant activity. 10 µL for UAE and 20 µL samples for SC-CO_2 were used in analysis. Applied procedure was similar to Sanchez-Moreno et al. (1997) described. Full description of the analysis can be found in Appendix A.7.

2.4.3 DETERMINATION OF TOTAL TOCOPHEROL CONTENT (TTC)

Tocopherol analysis were performed at Molecular Biology and Biotechnology Research Center- Central Laboratory- Metu by HPLC (VARIAN ProStar) with an Inertsil ODS-3 (250 x 4.6 mm, 5 µm) (VARIAN, A0396259x046) column at 295 nm. The mobile phase consisted Acetonitrile: Methanol (75:25) at a flow rate 1 mL/min at 40 °C column temperature. 50 µL sample was injected.

2.5 EXPERIMENTAL DESIGN

Matrix of the experimental design was determined by "Central Composite Rotatable Design" at three variables five factorial level. STATGRAPHICS© Centurion XV.II was used to fit data to response surfaces and determine parameters for optimized variables. The second order polynomial equation was used for analysis of variance.

$$Y := \beta_0 + \sum(\beta_i \cdot X_i) + \sum(\beta_{ii} \cdot X_i^2) + \sum(\beta_{ij} \cdot X_i \cdot X_j) \quad (5)$$

Y represents the experimental response, β_0, β_i, β_{ii}, and β_{ij} are regression coefficients of the model, and X_i and X_j are independent variables in coded values. The whole model includes linear, quadratic, and cross-product term.

$$Y := \beta_0 + \beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \beta_3 \cdot X_3 + \beta_{11} \cdot X_1^2 + \beta_{22} \cdot X_2^2 + \beta_{33} \cdot X_3^2 + \beta_{12} \cdot X_1 \cdot X_2 + \beta_{13} \cdot X_1 \cdot X_3 + \beta_{23} \cdot X_2 \cdot X_3 \quad (6)$$

The lowest and the highest values of each parameter is coded as –1.682 (-α) and +1.682 (+α), respectively. Specification of the lowest and the highest values determines the remaining part of the design. Coded value "0" stands for center values of the variables and repeated for experimental error. Factorial points are coded as ± 1. According to coded values (X_i) of second order three factorial five level experimental matrix, from stated ranges data points and variables are figured. Coded levels and variables for both of the extraction methods are demonstrated in following sections 2.5.1 and 1.5.2.

2.5.1 EXPERIMENTAL DESIGN FOR UAE

Ultrasonication time is represented by coded variable X_1, extraction temperature by X_2 and ethanol level % by X_3 (Table 2.1). Extraction ranges to be experimented are; for time 1-11 minute, for extraction temperature 20º-60ºC and 5-95 % for ethanol level %. In Table 2.2 all conditions of experimental set up is listed.

Table 2.1 Coded and Uncoded levels of Variables used for the CCRD of UAE.

		Coded Levels				
		-α (-1.682)	**-1**	**0**	**1**	**+α (+1.682)**
Code	**Independent Variables**	**Variable Levels**				
X_1	Ultrasonication Time (min)	1	3	6	9	11
X_2	Temperature (°C)	20	28	40	52	60
X_3	Ethanol Level (%)	5	23	50	77	95

Table 2.2 Matrix of Experimental Design for UAE

Exp. no.	Coded Parameters			Uncoded Parameters		
	X_1	X_2	X_3	U. Time min	Temperature ˚C	Ethanol Level %
1	1.682	0	0	11	40	50
2	1	1	1	9	52	77
3	1	1	-1	9	52	23
4	1	-1	1	9	28	77
5	1	-1	-1	9	28	23
6	0	1.682	0	6	60	50
7	0	0	1.682	6	40	95
8	0	0	-1.682	6	40	5
9	0	-1.682	0	6	20	50
10	-1	1	1	3	52	77
11	-1	1	-1	3	52	23
12	-1	-1	1	3	28	77
13	-1	-1	-1	3	28	23
14	-1.682	0	0	1	40	50
15	0	0	0	6	40	50
16	0	0	0	6	40	50
17	0	0	0	6	40	50
18	0	0	0	6	40	50
19	0	0	0	6	40	50
20	0	0	0	6	40	50
21	0	0	0	6	40	50
22	0	0	0	6	40	50
23	0	0	0	6	40	50

2.5.2 EXPERIMENTAL DESIGN FOR SC-CO_2 EXTRACTION

For SC-CO_2 extraction; extraction pressure is coded as X_1, extraction temperature as X_2, extraction time as X_3 (Table 2.3). Extraction ranges to be experimented are; for extraction pressure 148-602 bar, for extraction temperature 40º-60ºC and 10-60 min extraction time (Table 2.4).

Table 2.3 Coded and Uncoded Levels of Variables used for the CCRD of SC-CO_2 Extraction

		Coded Levels				
		-α (-1.682)	**-1**	**0**	**1**	**+α (+1.682)**
Code	**Independent Variables**	**Variable Levels**				
X_1	Pressure (bar)	148	240	375	510	602
X_2	Temperature (°C)	40	44	50	56	60
X_3	Time (min)	10	20	35	50	60

Table 2.4 Matrix of Experimental Design for SC-CO_2 Extraction

Exp. no.	Coded Parameters			Uncoded Parameters		
	X_1	X_2	X_3	Pressure bar	Temperature °C	Time min
1	1.682	0	0	602	50	35
2	1	1	1	510	56	50
3	1	1	-1	510	56	20
4	1	-1	1	510	44	50
5	1	-1	-1	510	44	20
6	0	1.682	0	375	60	35
7	0	0	1.682	375	50	60
8	0	0	-1.682	375	50	10
9	0	-1.682	0	375	40	35
10	-1	1	1	240	56	50
11	-1	1	-1	240	56	20
12	-1	-1	1	240	44	50
13	-1	-1	-1	240	44	20
14	-1.682	0	0	148	50	35
15	0	0	0	375	50	35
16	0	0	0	375	50	35
17	0	0	0	375	50	35
18	0	0	0	375	50	35
19	0	0	0	375	50	35
20	0	0	0	375	50	35
21	0	0	0	375	50	35
22	0	0	0	375	50	35
23	0	0	0	375	50	35

CHAPTER 3

RESULTS AND DISCUSSION

3.1 RAW AND ROASTED WHEAT GERM ANALYSIS

Particle size of the sample is an important parameter in the extraction. A properly reduced size increases the surface area and enhances the extraction. It was reported that the oil yield of SC-CO_2 extraction at 380 bar and 55ºC increased from 0.072 to 0.117 g oil/g wheat germ when particle size of the wheat germ reduced from 0.50mm to 0.35mm (Panfili et al., 2003). Furthermore, vitamin E yield of SC-CO_2 extraction at 206 bar, 40ºC with a 2 mL/min flow rate of CO_2 for 90 min increased from 16.19 mg/g to 18.38 mg/g as particle size of germ got finer from 2.1 mm to 0.5 mm (Ge et al., 2002a). However extensive grinding of germ to 0.22 mm, decreased vitamin E yield (from 18.38 to 10.70 mg/g) quite remarkably, possibly because of degradation due to increased area of contact with air. At high pressure SC-CO_2 extraction, smaller particles were also observed to form a hard "flake" causing supercritical fluid to pass through the space between the wall of the extraction cartridge and sample which causes a decrease in vitamin E yield (Ge et al., 2002a).

In order to determine if there was any need for grinding, sieve analysis were performed for both of un-milled and milled roasted wheat germs. Table 3.1 shows that 81% of un-milled germ was finer than 1.19mm and 12% finer than 0.6 mm. Un-milled roasted wheat germ had fine enough particle size for extraction (Table 3.1). On the other hand, 85% of milled germ was finer than 0.25mm (Table 3.2). Around the particle size which was reported to decrease vitamin E yield. Consequently it was decided not to grind roasted germ.

Moisture content of unroasted wheat germ was found to be 9.34%. After drying by spouted bed it was reduced to less than 1%. Ash contents of unroasted and roasted wheat germs were measured to be 4.21%, and 4.72%, respectively. Crude fat contents were 10.48% in unroasted wheat germ and 9.85% for roasted germ.

Table 3.1 Sieve Analysis of Roasted Wheat Germ

mesh #	mm	g wheat germ retained	% retained	% finer
16	1.19	29.6	19	81
18	1	62.22	40	41
20	0.841	14.63	9	32
25	0.707	17.5	11	21
30	0.595	13.24	9	12
pan		17.91	12	

Table 3.2 Sieve Analysis of Milled Roasted Wheat Germ

mesh #	mm	g wheat germ retained	% retained	% finer
60	0.25	24.65	16	85
80	0.177	53.81	35	50
100	0.149	32.7	21	29
120	0.125	22.6	15	14
140	0.105	13.37	9	5
pan		7.87	5	

3.2 ULTRASOUND ASSISTED EXTRACTION

Preliminary experiments were carried out in order to decide parameter levels. Ultrasound application (5-10 min) prior to solvent extraction increased the TPC of the extracts (Table 3.3). Extracts of roasted wheat germ yielded higher TPC

than unroasted wheat germ. Extraction with aqueous solvent at 40 ºC and a shorter time (1 min.) yielded higher amount of phenolics than previous study of Melikoğlu (2005)

These preliminary results clearly indicate the inadequacy of 1/10 solid to solvent ratio, in addition to pointing out the possible effects of temperature, ethanol level in solvent and extraction time. In order to eliminate the buffering effect of the bath in indirect application, direct sonication was applied to study the effect of sonication time, temperature and ethanol level. Dependent variables were; extraction yield %, total phenolic content and total antioxidant activity.

Table 3.3 TPC of Roasted Wheat Germ Extracts Obtained by Indirect UAE with or without Solvent Extraction

		Ultrasound Assisted Extraction		Solvent Extraction		
Solid/Solvent Ratio	**Ethanol Level (%)**	**Temp. (°C)**	**U.time (min)**	**Temp. (°C)**	**Time (min)**	**TPC[a]**
1/10	30	[d]	10	45	12	2.4
1/10	30 [d]	-	-	45	12	2.1
1/10	50	[d]	5	45	21	2.9
1/10	50 [d]	-	-	45	26	2.2
1/20	50	40	1	-	-	5.4
1/20	50 [d]	-	-	40	1	5.2
1/20 [b]	50	40	1	-	-	2.7
1/10	100	[d]	40	-	-	1.9[c]
1/10	100 [d]	-	-	-	-	0.8[c]

[a] Total Phenolic Content (mg GAE/g germ)
[b] Unroasted wheat germ
[c] by Melikoğlu (2005)
[d] ambient temperature

TPC and AA of the extracts were given both as per g extract and per g germ in Table 3.4. However, optimum extraction conditions for each dependent variable were modeled using "per g extract" data, since the aim of this study was to analyze and characterize the extract. Moreover "per g extract" expressing is technically more meaningful because wheat germ is a cheap by product and can be obtained easily.

Table 3.4 Yield of UAE, TPC and AA of Extracts of UAE

Exp. no.	U. Time (min)	Temp. (˚C)	Ethanol Level (%)	Yield[a]	TPC[b]	AA[c]	TPC[d]	AA[e]
1	11	40	50	33.0	16.4	68.6	5.4	22.6
2	9	52	77	32.6	17.7	165.3	5.8	53.9
3	9	52	23	51.2	12.2	40.7	6.3	20.9
4	9	28	77	17.6	16.1	97.2	2.8	17.1
5	9	28	23	36.4	13.6	82.8	5.0	30.1
6	6	60	50	43.8	15.4	80.6	6.7	35.3
7	6	40	95	17.3	21.8	88.7	3.8	15.3
8	6	40	5	53.8	12.8	55.3	6.9	29.7
9	6	20	50	22.6	15.9	79.8	3.6	18.0
10	3	52	77	34.4	16.8	92.1	5.8	31.7
11	3	52	23	51.0	12.4	53.9	6.0	27.5
12	3	28	77	15.6	15.5	125.9	2.4	19.6
13	3	28	23	35.4	15.0	85.8	5.3	30.4
14	1	40	50	40.2	17.9	71.1	7.2	28.6
15[f]	6	40	50	37.2 ±0.7	15.8 ±0.4	123.9 ±6.0	5.9 ±0.1	46.1 ±2.1

[a] % (g extract ×100/g germ)
[b] Total Phenolic Content (mg GAE/g extract)
[c] Antioxidant Activity (mg scavenged $DPPH^{\cdot}$/g extract)
[d] Total Phenolic Content (mg GAE/g germ)
[e] Antioxidant Activity (mg scavenged $DPPH^{\cdot}$/g germ)
[f] Average of 9 replications at center point conditions.

The coefficients of the second order models for each response were evaluated by nonlinear regression option of Statgraphics© and fitted to equation (5). The p-value of each parameter represents the statistical significance of the parameter. As p-value approaches zero, the significance of the parameter increases. In most cases smaller p-value than 0.05 is accepted to be statistically significant. The models were found to represent the experimental data quite well (r^2: 0.71-0.96) Detailed expression of p-values for coefficients and r^2 of the models is given in Appendix C.1.

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

Table 3.5 Estimated Coefficients of the Second Order Response Model for UAE

		Estimate		
Term	**Coefficient**	**Yield[a]**	**TPC[b]**	**AA[c]**
constant	β_0	37.2552*	15.8515*	123.414*
X_1	β_1	-0.7841	-0.2011	1.76775
X_2	β_2	7.3116*	-0.1470	-2.7922
X_3	β_3	-9.9036*	2.04458*	20.0385*
X_1^2	β_{11}	-0.3726	0.08946	-14.541*
X_2^2	β_{22}	-1.5747*	-0.4320	-10.896
X_3^2	β_{33}	-0.7509	0.13719	-13.790*
$X_1 \times X_2$	β_{12}	-0.575	0.1875	11.45
$X_1 \times X_3$	β_{13}	-0.125	0.3875	7.585
$X_2 \times X_3$	β_{23}	0.425	0.8775	13.52

[a] % (g extract ×100/g germ)
[b] Total Phenolic Content (mg GAE/g extract)
[c] Antioxidant Activity (mg scavenged $DPPH^{\cdot}$/g extract)
* Significant at $p \leq 0.05$.

3.2.1 EXTRACTION YIELD

Figure 3.1 shows that UAE time did not significantly affect the extraction yield (%) at low extraction temperatures. Previously, Usaquen-Castro et al. (2006) observed that ultrasound exposure times lower than 25 min. do not cause significant effects on extraction yield compared to the best performance of extraction yield (37.5%) for 90 min. UAE. The authors concluded that significant effects ($p \leq 0.05$) of ultrasound exist at a minimum exposure time 45 min. Similar to their conclusions, the in effect of shorter ultrasound exposure times (1-11 min) were also found not to be significant ($p \leq 0.05$) on extraction yield in the present study.

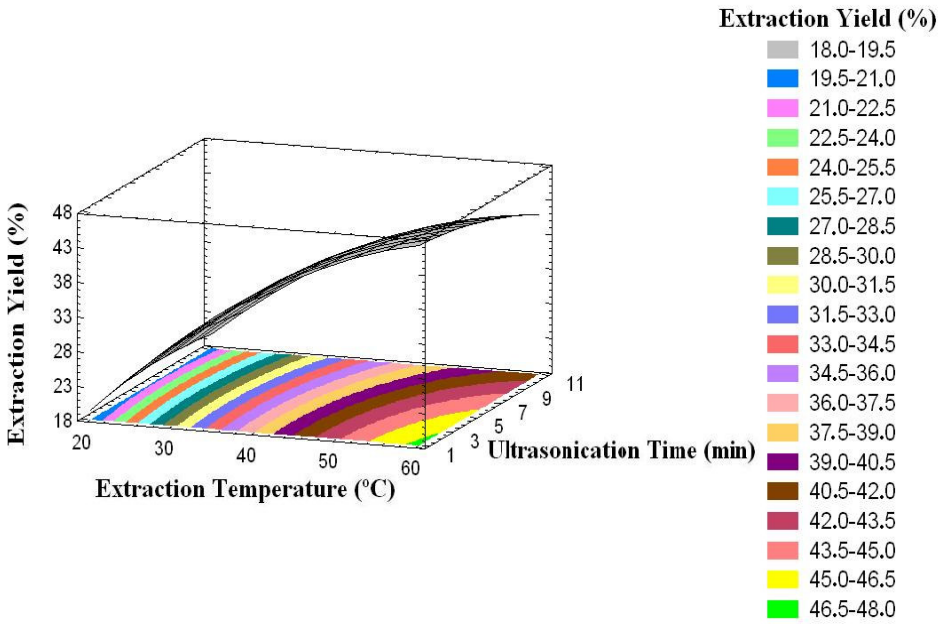

Figure 3.1 Effects of Temperature & Ultrasonication Time on UAE Yield (%) (Ethanol Level = 50 %)

Increasing temperature decreased the viscosity of the extracts while enhancing the diffusivity of them in to the solvent and increasing the extraction yield. Figure 3.1 and 3.2 shows increase of yield (%) with temperature.

Ethanol level (%) of the solvent significantly ($p \leq 0.05$) affected the extraction yield (Figure 3.2 & 3.3). Extraction yield decreased with increasing ethanol level (%). It is possible to explain the inverse proportionality by the extraction of water-soluble albumin. The albumin was reported to be the predominant protein fraction, accounting for 34.5% of the total proteins, in wheat germ. The salt-soluble and alkali-soluble fractions were 15.6 and 10.6 %, respectively. However, the alcohol soluble fraction (prolamine) was only 4.6% (Zhu et al., 2006). The results of this study, indicated that germ proteins are better extracted with lower levels of ethanol and this enhanced the extraction yield (%).

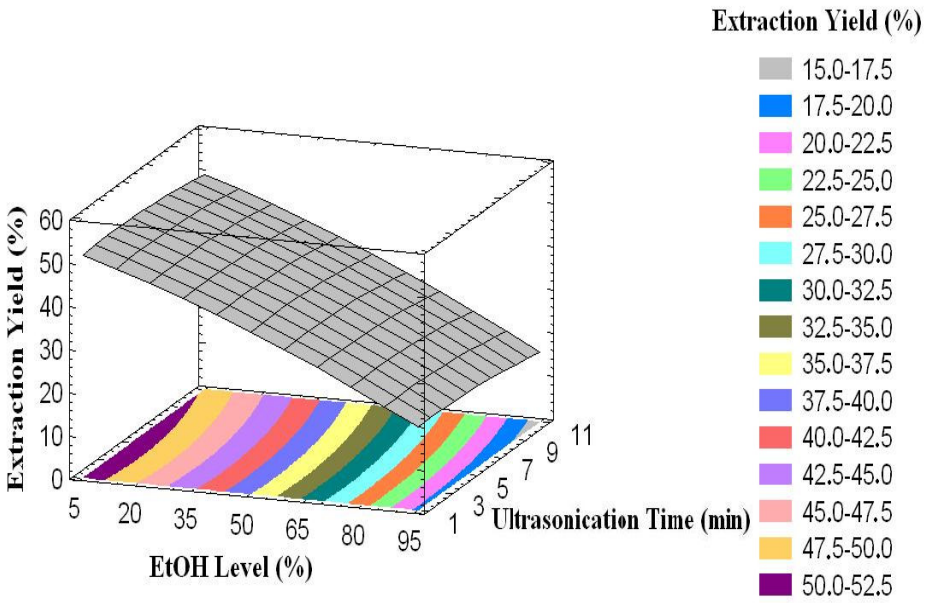

Figure 3.2 Effects of Ethanol Level & Ultrasonication Time on UAE Yield (%) (Temperature = 40 ºC)

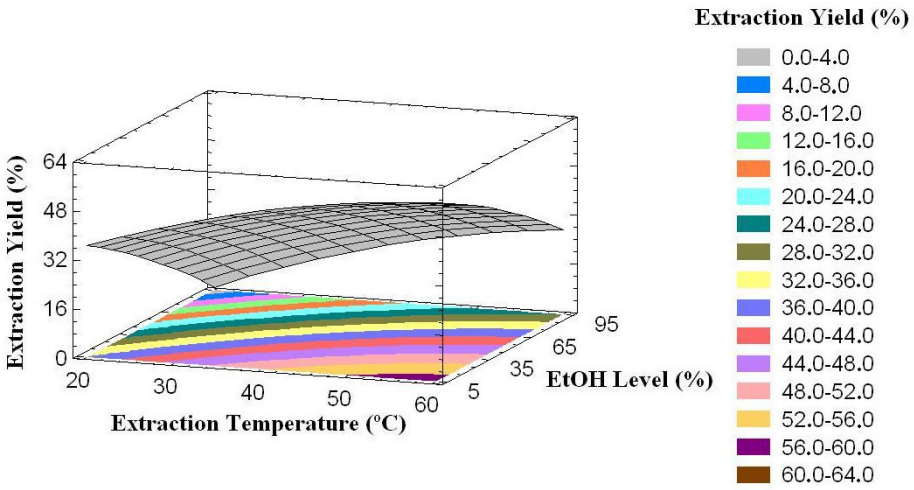

Figure 3.3 Effects of Temperature & Ethanol Level on UAE Yield (%)
(Ultrasonication Time= 6 min)

3.2.2 TOTAL PHENOLIC CONTENT OF THE EXTRACTS OBTAINED BY UAE

Longer sonication times decreased the TPC (mg GAE/ g extract) of the extracts at all temperatures (Figure 3.4). At 50% ethanol level, temperature appears to have a parabolic effect on TPC with a maximum at 40ºC, but the absolute value of the effect is quite small.

Longer sonication times decreased the TPC (mg GAE/ g extract) of the extracts at low levels (5-50%) of ethanol (Figure 3.5), while at higher ethanol levels opposite effect of sonication time was observed.

Increasing extraction temperature enhanced the solubility of phenolics in ethanol and increased the TPC of the extracts at 6 min extraction time (Figure 3.6), although the effect diminishes at around 40% ethanol and is in the reverse

direction at lower ethanol levels. Wang et al. (2008) have also concluded that there was a significant increase in TPC of the wheat bran extracts over the temperature range 25º-75ºC.

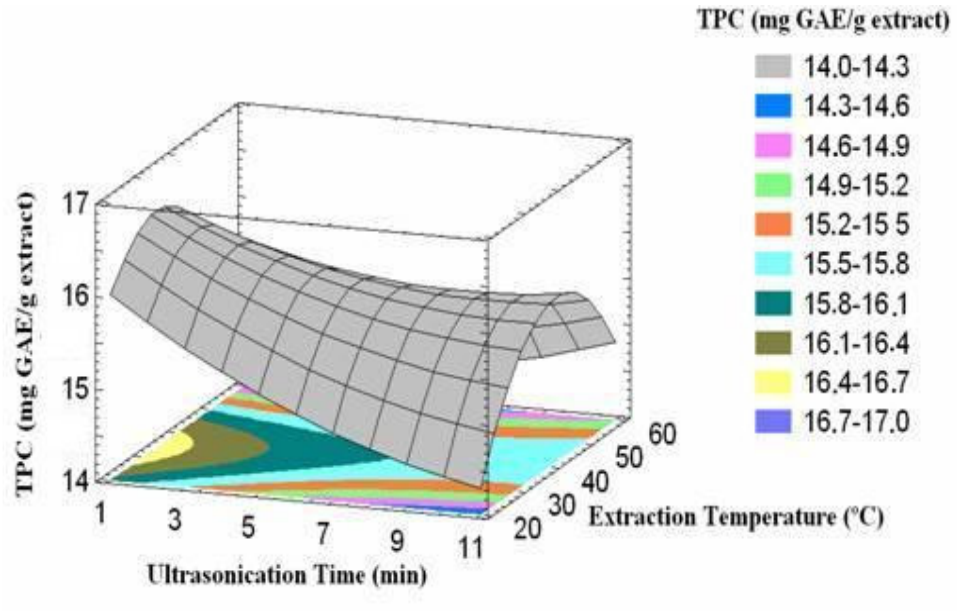

Figure 3.4 Effects of Ultrasonication Time and Extraction Temperature on TPC (mg GAE /g extract) of the extracts for UAE
(Ethanol Level = 50%)

In Figures 3.5 & 3.6, effects of ethanol levels on TPC of the extracts are presented clearly. On "per g extract" basis, at higher levels of ethanol TPC of the extracts were observed to increase as the extraction yield decreased. On the contrary, on the basis of "per g germ", a maximum TPC of the extracts were observed at low ethanol level (30%) and short ultrasonication time (1 min).

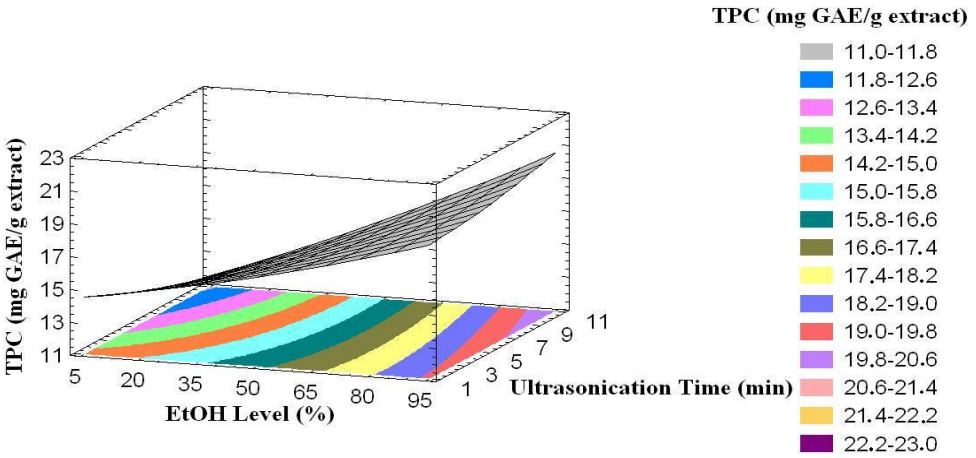

Figure 3.5 Effects of Ethanol Level (%) and Ultrasonication Time on TPC (mg GAE /g extract) of the extracts for UAE

(Extraction Temperature = 40 ºC)

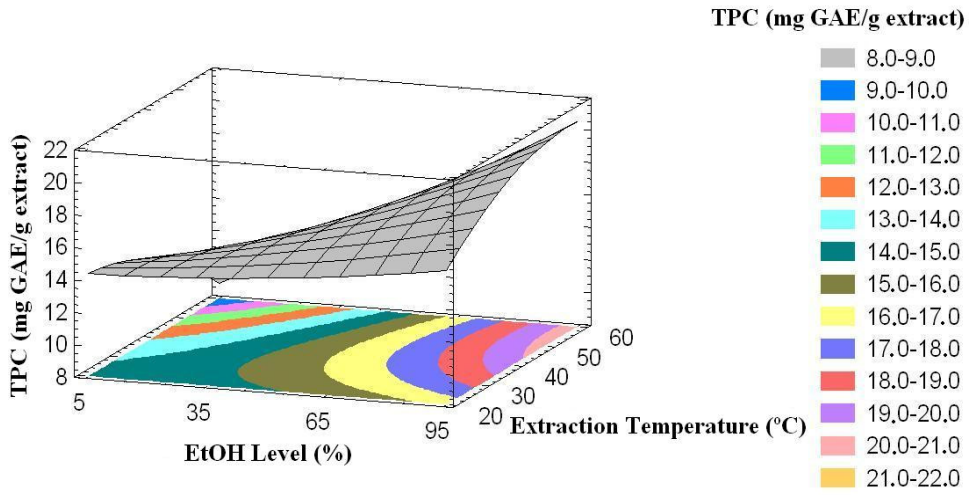

Figure 3.6 Effects of Ethanol Level and Extraction Temperature on TPC (mg GAE /g extract) of the extracts for UAE

(Ultrasonication Time= 6 min)

Previously, it has been demonstrated that ultrasonic waves improved the extraction level of calcium, potassium and magnesium (Arruda et al., 2003) which are remarkably found in wheat germ (Table 1.8). According to their statement and Figures 3.5 & 3.6, it was concluded that at low levels of ethanol, longer ultrasound exposure increased the extraction of proteins, minerals and carbohydrates from wheat germ. This increase in yield surpassed the extraction of phenolics and decreased TPC of the extracts.

3.2.3 ANTIOXIDANT ACTIVITY OF THE EXTRACTS OBTAINED BY UAE

In their study Usaquen-Castro et al. (2006) stated that at the studied frequency (35 kHz), TPC of the extracts was enhanced as ultrasound exposure time increased to 45 min., however AA of the extracts were reduced 50 % compared to non-sonicated sample. In same manner, longer sonication times than caused a decrease in AA of the wheat germ extracts (Figure 3.7&3.8). Excessive exposure to sonication power and air could be decreasing AA of the extracts. Since not only phenolics, but also tocopherols and carotenoids affect the AA of the extracts.

In the temperature range 20º-100ºC, α-tocopherol activity was reported to increase with temperature by Marinova and Yanishlieva (2003). As the extraction of tocopherols and phenolics were enhanced by studied temperature range (20-60ºC), the AA of the wheat germ extracts increased with temperature (Figure 3.7&3.9).

Krings et al. (2000) suggested that main antioxidative components of wheat germ possessed polar characteristics since ethanol was the best extraction solvent. Along with sonication and temperature, increasing ethanol level (up to about 75%) increased the AA of the extracts (Figure 3.8&3.9).

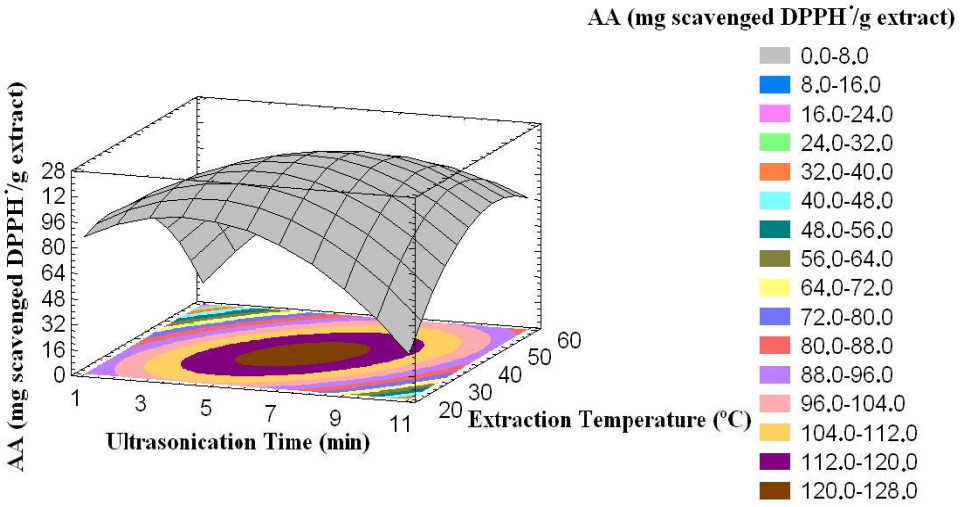

Figure 3.7 Effects of Ultrasonication Time and Extraction Temperature on AA of the extracts for UAE (mg Scavenged DPPH˙ / g extract)
(Ethanol Level = 50%)

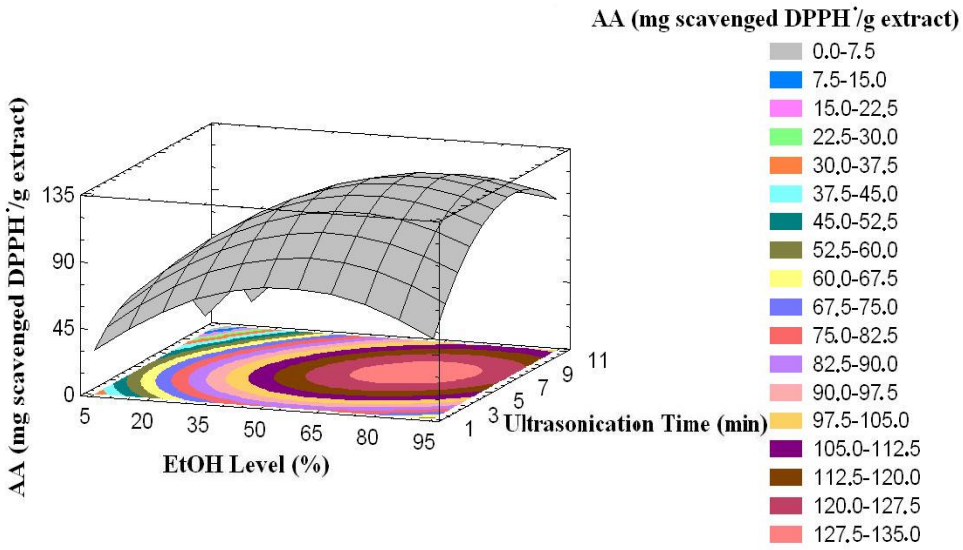

Figure 3.8 Effects of Ethanol Level and Ultrasonication Time on AA of the extracts for UAE (mg Scavenged DPPH˙ / g extract)
(Extraction Temperature = 40 ºC)

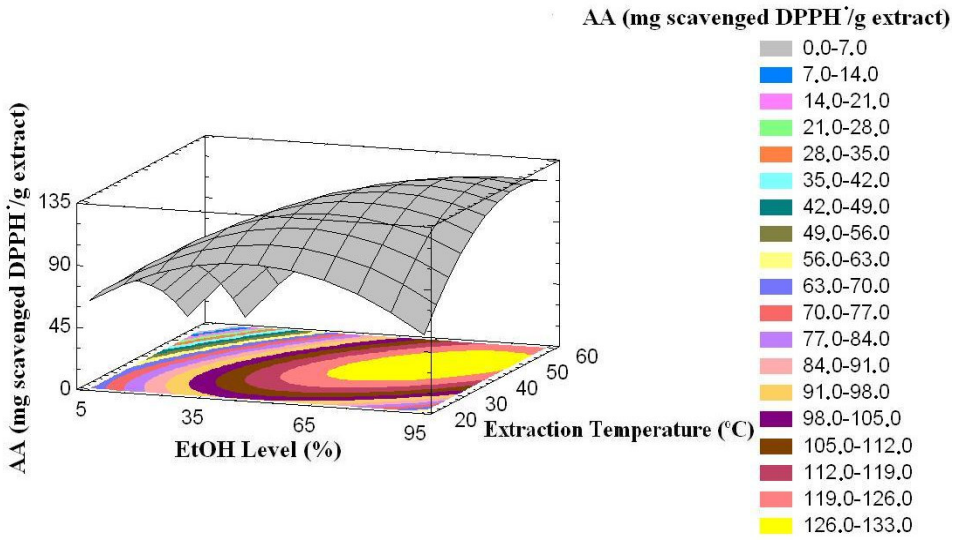

Figure 3.9 Effects of Ethanol Level and Extraction Temperature on AA of the extracts for UAE (mg Scavenged DPPH˙ / g extract)
(Ultrasonication Time = 6 min)

3.2.4 TOCOPHEROL CONTENT OF THE EXTRACTS OBTAINED BY UAE

The extract obtained at the optimum conditions of AA (9 minutes sonication with 95 % ethanol level at 58ºC) was analyzed for its TTC. δ-tocopherol was not detected in extract. "β- & γ-tocopherol" content was found to be 0.369 mg/g extract (0.104 mg /g germ). α-tocopherol content was 0.801 mg/g extract (0.226 mg/ g germ). A total 1.17 mg/g extract (0.33 mg tocopherol / g germ) was recorded, which compares favorably with the more dependable ones of the literature values. Further comments on this point can be found in Section 3.3.4.

3.2.5 OPTIMUM EXTRACTION CONDITIONS AND VALUES

By setting coefficients precisely to illustrate response surfaces, factorials were optimized and adequate conditions were set for maximum values of each dependent variable. Table 3.6 gives optimum values for factors and variables. Beside discrete set of conditions, one set of factors that maximize all of the three dependent variables at the same time, was determined by "Multiple Response Optimization" (Table 3.7, 3.8 & 3.9).

Optimum UAE conditions for extraction yield was 3 min, 60 ºC and 5% level of ethanol. At those conditions the extraction yield increased to 60%, since the extracted amount of minerals, water soluble proteins and carbohydrates were enhanced.

Table 3.6 Optimum Values of Responses and Factors for UAE

Response	Optimum Value	Coded Levels			Uncoded Levels		
		X_1	X_2	X_3	U. Time (min)	Temp. (°C)	Ethanol Level (%)
Yield[a]	59.7	-1.094	1.682	-1.682	3	60	5
TPC[b]	21.5	1.682	1.410	1.530	11	57	91
AA[c]	140.8	1.084	1.485	1.682	9	58	95
TPC[d]	7.2	-1.682	0.841	-0.407	1	50	29
AA[e]	56	0	0.505	0.260	6	46	43

[a] % (g extract ×100/g germ)
[b] Total Phenolic Content (mg GAE/g extract)
[c] Antioxidant Activity (mg scavenged DPPH˙/g extract)
[d] Total Phenolic Content (mg GAE/g germ)
[e] Antioxidant Activity (mg scavenged DPPH˙/g germ)

TPC of the extracts, obtained at optimum conditions (11 min, 57ºC, 91% ethanol level), was 21.5 mg GAE/g extract. Wang et al. (2008) optimized the extraction of phenolics compounds from wheat bran and observed a maximum TPC (3.12 mg GAE/ g wheat bran) of the extracts for 25 min. ultrasonication, at 64% ethanol level and 60ºC. Compared to the TPC of wheat bran extracts, on "per g sample basis" the TPC of wheat germ extracts was almost two times higher.

Table 3.7 Coded Optimum Values for Factors
(Multiple Response Optimization for UAE)

Factor	Coded Value
X_1	0.79473
X_2	1.54484
X_3	1.19367

Table 3.8 Uncoded Optimum Values for Factors
(Multiple Response Optimization for UAE)

Factor	Uncoded Value
Ultrasonication Time (min)	8 min
Extraction Temperature (ºC)	59ºC
Ethanol Level %	82 %

UAE conditions for optimum AA (140.8 mg scavenged DPPH˙/ g extract) of the extracts was 9 min ultrasonication time, at 58ºC and 95% ethanol level. This set of conditions was similar to the one obtained for optimum TPC of the extracts. With increasing ethanol level, extraction yield of proteins, minerals and carbohydrates decreased. However, extraction of phenolics and tocopherols was enhanced and AA of the extracts increased.

Conditions obtained by multiple response optimization of UAE were 8 min ultrasonication time, at 59ºC and 82% ethanol level. Extraction yield was 31%. TPC and AA of the extracts were 19.3 mg GAE/g extract, 135.8 mg scavenged DPPH˙/g extract, respectively (Table 3.9).

Table 3.9 Optimum Values of Responses
(Multiple Response Optimization for UAE)

Response	Optimum Value
Yield[a]	31.0
TPC[b]	19.3
AA[c]	135.8

[a] % (g extract ×100/g germ)
[b] Total Phenolic Content (mg GAE/g extract)
[c] Antioxidant Activity (mg scavenged DPPH˙/g extract)

3.3 SUPERCRITICAL CO_2 EXTRACTION

Effects of extraction pressure, temperature and time on extraction yield %, TPC and AA of germ oil was studied and expressed as "per g extract" (Table 3.10). There was a high statistically significant relationship between fitted models and data (r^2: 0.84-0.98) (Appendix C.2).

The coefficients of the second order models were evaluated by;

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

Coefficients of models for each of the dependent variable are given in Table 3.10.

Table 3.10 Yield of SC-CO_2 Extraction, TPC and AA of the Extracts of SC-CO_2 Extraction

Exp. no.	Press. (bar)	Temp. (˚C)	Time (min)	Yield[a]	TPC[b]	AA[c]	TPC[d]	AA[e]
1	602	50	35	8.21	2.6	18.2	0.21	7.1
2	510	56	50	8.43	4.5	21.7	0.38	8.7
3	510	56	20	8.17	4.0	28.7	0.33	11.6
4	510	44	50	8.95	3.9	31.1	0.35	13.3
5	510	44	20	8.15	3.2	31.8	0.26	12.4
6	375	60	35	8.50	4.7	24.8	0.40	9.6
7	375	50	60	8.51	3.4	36.2	0.29	14.7
8	375	50	10	6.21	3.9	56.5	0.24	13.9
9	375	40	35	8.01	2.8	37.9	0.22	14.5
10	240	56	50	7.39	4.4	26.4	0.33	10.6
11	240	56	20	3.98	6.6	54.6	0.26	11.1
12	240	44	50	7.27	3.7	39.9	0.27	13.8
13	240	44	20	4.04	6.4	59.4	0.26	12.0
14	148	50	35	2.76	5.4	52.9	0.15	6.8
15[f]	375	50	35	8.3 $\pm$0.10	3.7 $\pm$0.1	35.6 $\pm$1.3	0.31 +0.01	14.1 $\pm$0.2

[a] % (g extract ×100/g germ)
[b] Total Phenolic Content (mg GAE/g extract)
[c] Antioxidant Activity (mg scavenged $DPPH^{\cdot}$/g extract)
[d] Total Phenolic Content (mg GAE/g germ)
[e] Antioxidant Activity (mg scavenged $DPPH^{\cdot}$/g germ)
[f] Average of 9 replications at center point conditions

Pressure and time were significantly effective ($p \leq 0.05$.) while temperature had almost no effect on extraction yield of oil from wheat germ. All three factorials

were identified to be significantly effective on TPC mg/g extract, however only temperature showed a positive effect. AA of the extracts were negatively affected by both pressure, temperature and time.

Table 3.11 Estimated Coefficients of the Second Order Polynomial Equation for SC-CO_2 Extraction

		Estimate		
Term	**Coefficient**	**Yield[a]**	**TPC[b]**	**AA[c]**
constant	β_0	8.33022*	3.7389*	35.624*
X_1	β_1	1.47807*	-0.75551*	-9.17173*
X_2	β_2	0.028123	0.40273*	-3.87585*
X_3	β_3	0.84705*	-0.33499*	-6.56156*
X_1^2	β_{11}	-0.98737*	0.25959	-0.25864
X_2^2	β_{22}	-0.00802	0.16713	-1.7464
X_3^2	β_{33}	-0.32445	0.11976	3.55232
$X_1 \times X_2$	β_{12}	-0.07	0.058	0.70425
$X_1 \times X_3$	β_{13}	-0.6975*	0.76125	5.00325*
$X_2 \times X_3$	β_{23}	-0.045*	0.03925*	-1.87125*

[a] % (g extract ×100/g germ)
[b] mg GAE/g extract
[c] mg scavenged $DPPH^{\cdot}$/g extract
*Significant at $p \leq 0.05$.

3.3.1 EXTRACTION YIELD

Oil solubility of soybean was reported to increase as pressure increased because CO_2 is dense at high pressures and causes a decrease in yield (King and Bott,

1993). As pressure increased, yield increased up to approximately 450 bar (Figure 3.10 and 3.11). This is also due to the increased density of CO_2 with pressure. After 450 bar increase in yield was not significant.

Temperature did not have a significant effect on extraction yield (Figure 3.10 & 3.12).

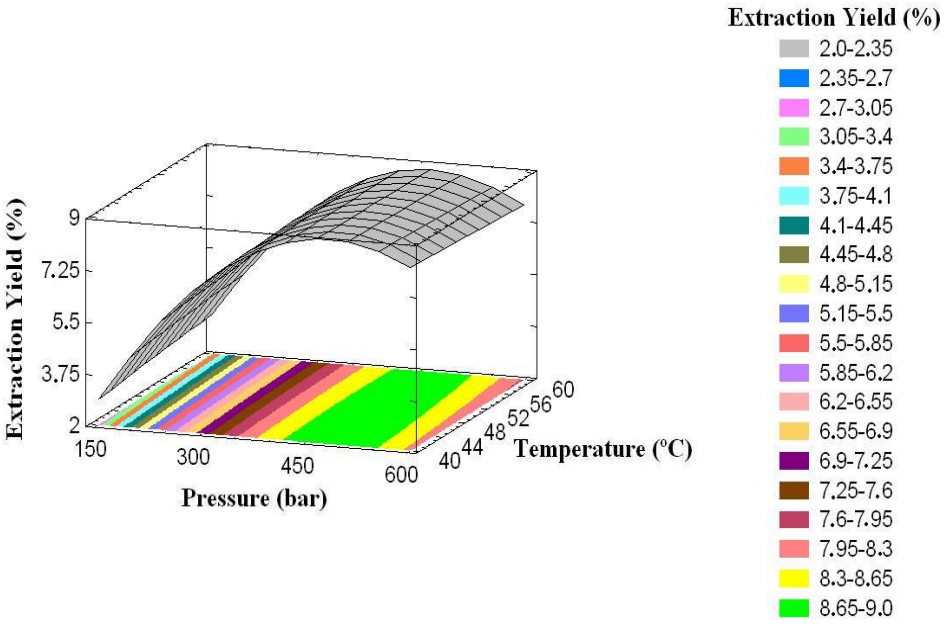

Figure 3.10 Effects of Pressure and Temperature on SC-CO_2 Extraction Yield (%)
(Time = 35 min)

Time had a significant effect (Table 3.11) on extraction yield of oil from roasted wheat germ (Significant at $p \leq 0.05$). Yield increased with time especially at low pressures up to about 45 minutes. Crude fat content of roasted wheat germ was measured as 9.85% and 45 minutes was enough to extract almost all of the oil in germ.

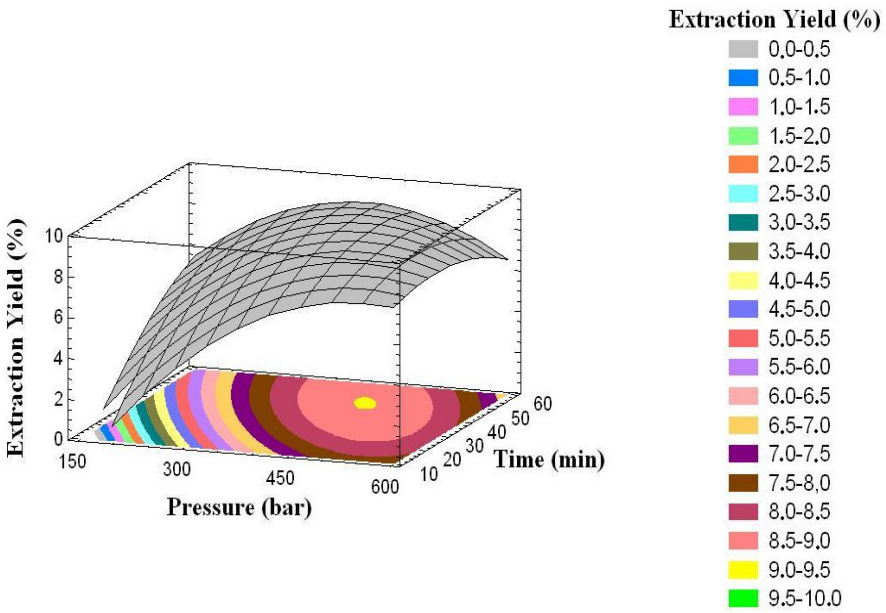

Figure 3.11 Effects of Pressure and Time on SC-CO_2 Extraction Yield (%)

(Temperature = 50 ºC)

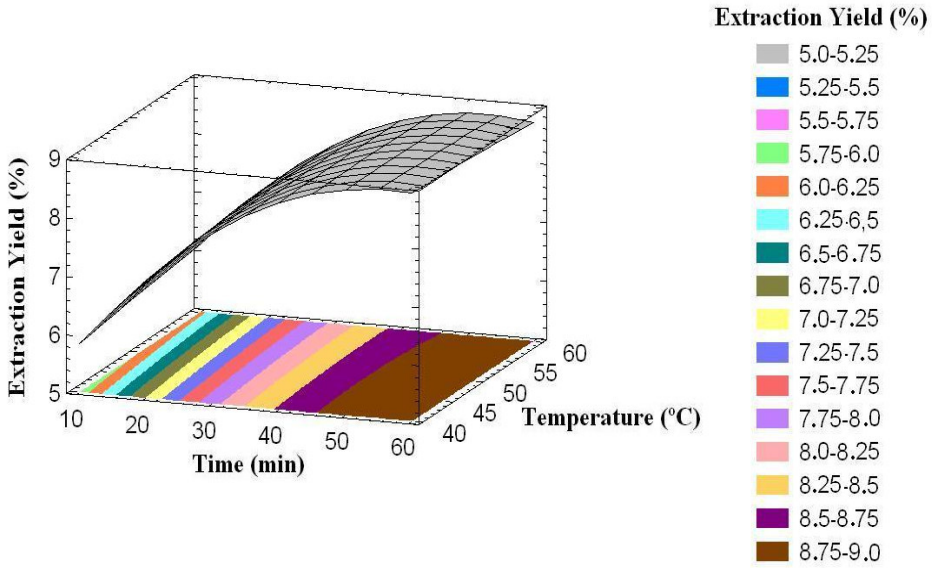

Figure 3.12 Effects of Time and Temperature on SC-CO_2 Extraction Yield (%)

(Pressure = 375 bar)

3.3.2 TOTAL PHENOLIC CONTENT OF THE EXTRACTS OBTAINED BY SC-CO_2 EXTRACTION

Figure 3.13 shows that TPC of the extracts decreased with increasing pressure. It is known that solubility of phenolics is low in SC-CO_2 (Hasbay-Adil et al., 2007) but increases with increasing pressure. Although amount of phenolic compound extracted from wheat germ increased with pressure, TPC (mg GAE/g extract) of the extracts decreased because extraction yield of oil increased at the same time (Figure 3.10 & 3.13). Pressure had a larger effect on solubility of oil of the extracts than the solubility of the phenolic compounds therefore, their concentrations in the extracts decreased with pressure.

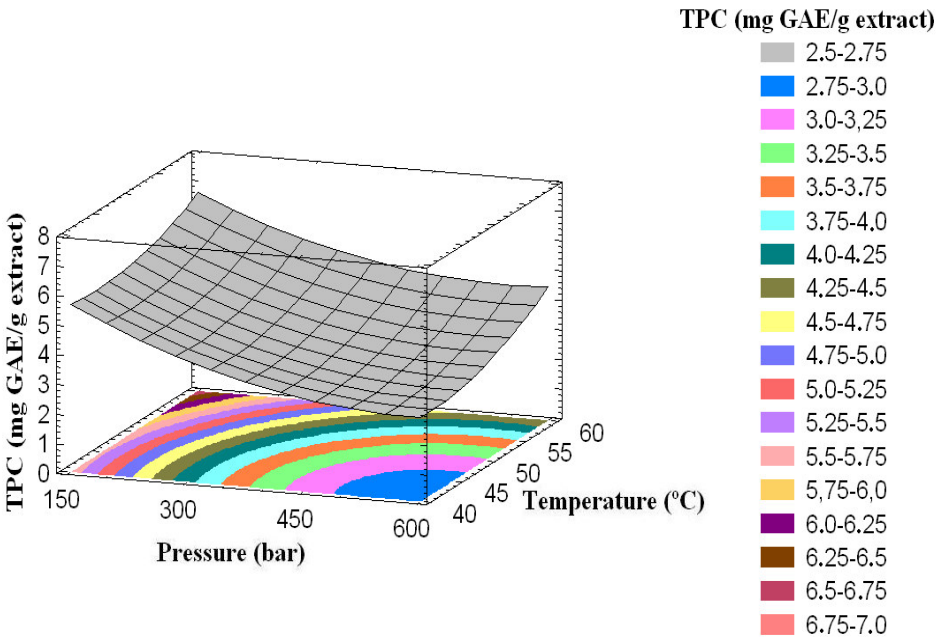

Figure 3.13 Effects of Pressure and Temperature on TPC of SC-CO_2 Extracts (Time = 35 min)

On the other contrary, since temperature did not have a significant effect on extraction yield (Table 3.11, Figure 3.10 & 3.12), amount of phenolic compounds and TPC of the extracts with increased temperature (Hasbay-Adil et al., 2007). Increase with temperature is shown in Figures 3.13 & 3.14.

Time significantly affected and increased extraction yield up to 45 minutes. Therefore, for short extraction times, TPC of the extracts decreased with pressure but for long extraction times, TPC of the extracts increased with pressure (Figure 3.14).

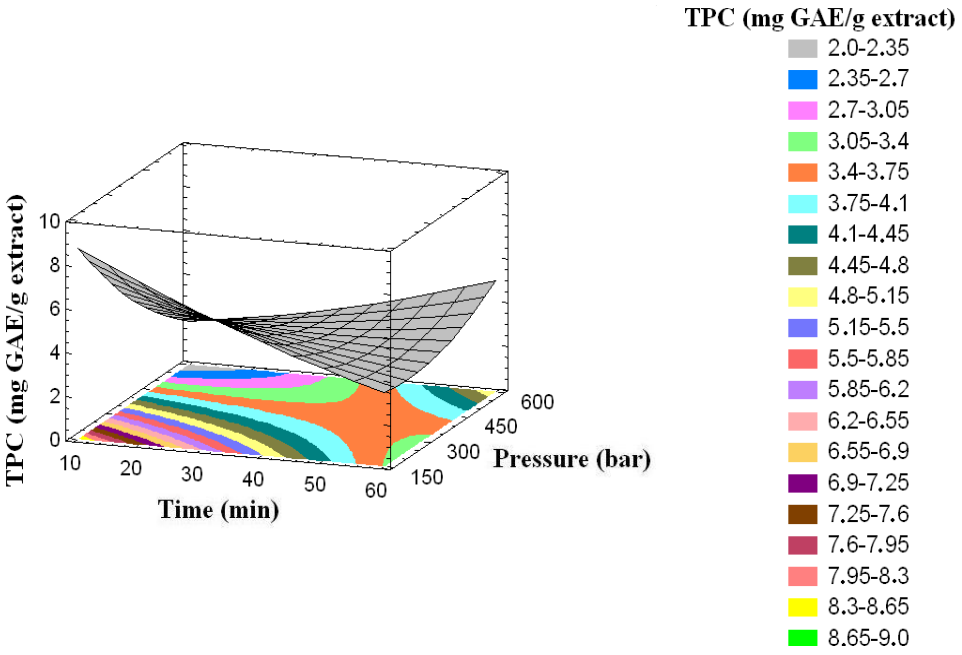

Figure 3.14 Effects of Time and Pressure on TPC of SC-CO_2 Extracts (Temperature = 50 ºC)

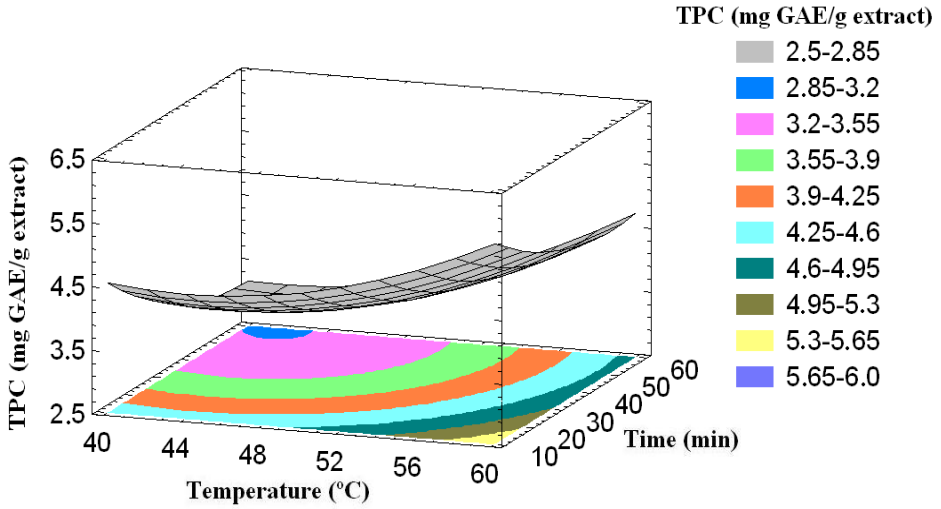

Figure 3.15 Effects of Temperature and Time on TPC of SC-CO_2 Extracts (Pressure = 375 bar).

3.3.3 ANTIOXIDANT ACTIVITIES OF EXTRACTS OBTAINED BY SC-CO_2 EXTRACTION

Pressure, temperature and time had a significant effect ($p \leq 0.05$) on AA (Table 3.10). AA of the extracts decreased with all three parameters.

Change in AA of the extracts was affected by the proportions of all antioxidants (phenolic compounds, tocopherols and carotenoids) and oil present in the extracts. The carotenoid content of the extracts was expected to be low since it was reported that maximum tocopherol was extracted much earlier than carotenoids from germ oil (Panfili et al., 2003).Pressure affected AA of the extracts same as TPC (Figure 3.13, 3.16 & 3.17). The AA of responses which are not similar to TPC, are believed to be resulted from the differences in the extraction mechanism of vitamin E compared to phenolic compounds.

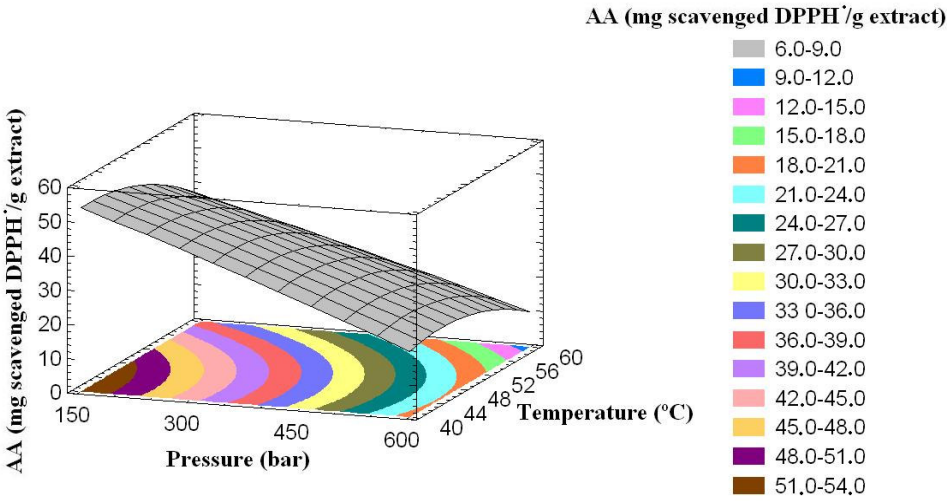

Figure 3.16 Effects of Pressure and Temperature on AA of SC-CO_2 Extracts (Time = 35 min)

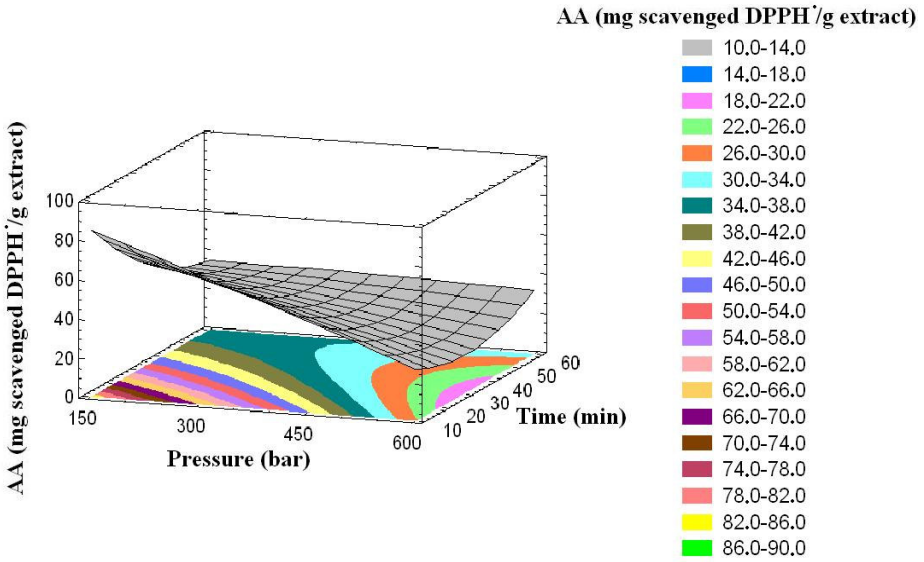

Figure 3.17 Effects of Pressure and Time on AA of SC-CO_2 Extracts (Temperature = 50 ºC)

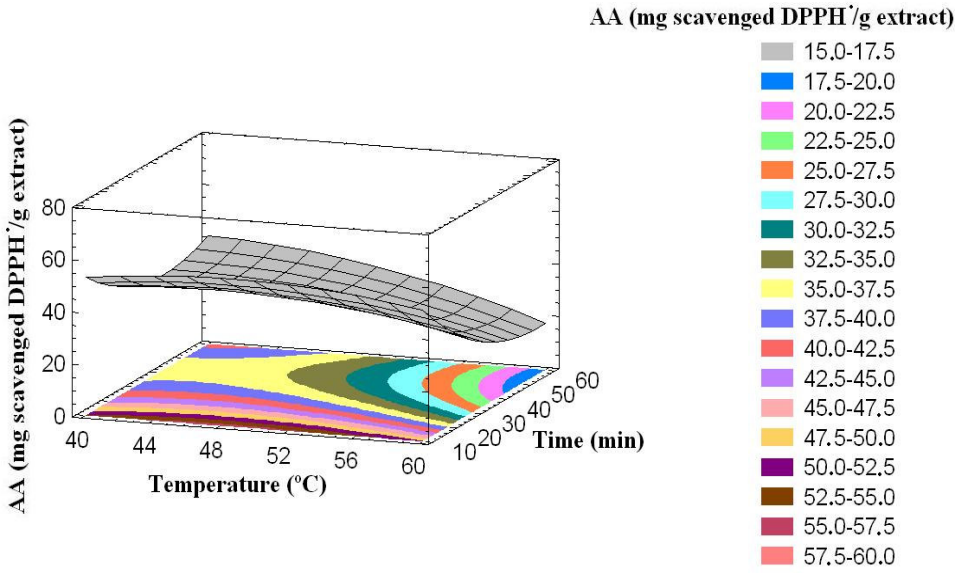

Figure 3.18 Effects of Temperature and Time on AA of SC-CO_2 Extracts (Pressure = 375 bar)

3.3.4 TOCOPHEROL CONTENT OF THE EXTRACTS

The extracts were analyzed to determine α-, β-, γ-, δ- tocopherol contents (Appendix B.2). Table 3.12 gives the total tocopherol contents of the extracts.

Maximum tocopherol content (g/g extract) was observed at 240 bar, 44-56ºC, for 20 minute extraction. This was parallel with maximum conditions (240 bar, 44ºC, 20 min) found for AA of SC-CO_2 extracts (Table 3.10).

Table 3.12 TTC of the Extracts obtained by SC-CO_2 Extraction

Exp. no.	Press. (bar)	Temp. (˚C)	Time (min)	TTC[a]	TTC[b]
1	602	50	35	2.784	0.229
2	510	56	50	3.192	0.269
3	510	56	20	3.780	0.309
4	510	44	50	2.961	0.265
5	510	44	20	3.252	0.265
6	375	60	35	3.280	0.279
7	375	50	60	2.810	0.239
8	375	50	10	4.806	0.298
9	375	40	35	3.537	0.283
10	240	56	50	2.648	0.196
11	240	56	20	7.142	0.284
12	240	44	50	3.080	0.224
13	240	44	20	7.040	0.284
14	148	50	35	3.845	0.106
15[c]	375	50	35	3.531±0.046	0.294±0.003

[a] Total Tocopherol Content (mg total tocopherol/ g extract)
[b] Total Tocopherol Content (mg total tocopherol/ g germ)
[c] Average of 9 replications at center point conditions.

Total tocopherol (or α-tocopherol) contents of wheat germ oil reported in the literature show a great variation. There are 1 to 2 orders of magnitude differences between them as grouped and shown in Table 3.13.

Table 3.13 Comparison of TTC of Wheat Germ Oil Extracted with Different Extraction Methods

Extraction Method	TTC		Reference
	mg/g germ	mg/g oil	
-	-	1.5[a]	NAL, 2008
-	0.28	2.6	Belitz and Grosch, 1987
SC-CO_2 extraction 380 bar, 55°C, 75 min	0.32[b]	2.9	Panfili et al., 2003
Soxhlet Extraction Petroleum Ether, 9h	0.29[b]	2.3	Panfili et al., 2003
SC-CO_2 extraction 140 bar, 80°C	-	26.86	Eisenmenger et al., 2006
SC-CO_2 extraction 276 bar, 40°C, 90 min	21.79[c]	-	Ge et al, 2002a
SC-CO_2 extraction 345 bar, 43°C, 90 min	23.07[c]	-	Ge et al., 2002b
SC-CO_2 extraction 150 bar, 40°C, 3h	33.33[b]	416.7	Molero Gomez et al., 2000
Soxhlet Extraction, Hexane, 16h	19.95[b]	232	Molero Gomez et al., 2000
SC-CO_2 extraction 240 bar, 56°C, 20 min	0.31	7.14	Present work
UAE 9 min, 58°C, 95% ethanol	0.33	1.17	Present work

[a] mg α-tocopherol
[b] the value was calculated by using the extraction yield reported by the authors
[c] reported as mg/g as the authors

It is clear that SC-CO_2 extraction is a better method than solvent extraction for tocopherols. Tocopherol content obtained at 240 bar, 44-56ºC, for 20 minute extraction (0.31 mg/g germ) was in good agreement with the ones reported by NAL(2008), Belitz and Grosch (1987) and Panfili et al. (2003).

The maximum tocopherol content, 7.14 mg/g oil obtained in this study was

higher than 2.9 g/g oil reported by Panfili et al. (2003), because of the higher oil yield at their extraction conditions.

3.3.5 OPTIMUM EXTRACTON CONDITIONS AND VALUES

By setting regression coefficients precisely to illustrate response surfaces, factorials were optimized and adequate conditions were set for maximum values of each dependent variable. Table 3.14 gives optimum values for factors and variables.

Beside discrete set of conditions, one set of factors that maximize all of the three dependent variables at the same time was determined by "Multiple Response Optimization" on "per g extract" base (Table 3.15, 3.16 & 3.17).

Optimum extraction conditions for extraction yield were 442 bar, 40 ºC and 48 min. The extraction yield at those conditions was 9%. This amount almost includes all of the crude fat found in roasted wheat germ (9.85%). Gomez et al. (2000) stated the optimum extraction yield (8%) at 150 bar, 40ºC for 3 hours extraction. At those conditions vitamin E content of the oil was maximized, as well. Comparison of optimum conditions obtained by present study and Gomez et al. at constant temperature, shows that higher pressure is required to obtain same extraction yield in a shorter time.

Optimum conditions for TPC (9.55 mg GAE/g extract) of the extracts were found as 148 bar, 59ºC and 10 min. Optimum extraction conditions for AA of the extracts were found to be similar to the optimum conditions for TPC of the extracts, as 148 bar, 48 ºC and 10 min.

It could be concluded that TPC and AA of the extracts were high at the extraction

conditions where the extraction yield was low. That was because of the diluting effect of oil, at high extraction yields. However, the optimum extraction conditions (especially pressure), found using "per g germ basis" for TPC and AA of the extracts, were more comparable with the ones found for yield; as expected.

Table 3.14 Optimum Values of Responses and Factors for SC-CO_2 Extraction

Response	Opt. Value	Coded Levels			Uncoded Levels		
		X_1	X_2	X_3	Press. (bar)	Temp. (˚C)	Time (min)
Yield[a]	9	0.493	-1.682	+0.892	442	40	48
TPC[b]	9.55	-1.682	1.518	-1.682	148	59	10
AA[c]	86	-1.682	-0.416	-1.682	148	48	10
TPC[d]	0.44	0.630	1.682	1.682	460	60	60
AA[e]	3.57	-0.104	-1.682	1.682	361	40	60

[a] % (g extract ×100/g germ)
[b] Total Phenolic Content (mg GAE/g extract)
[c] Antioxidant Activity (mg scavenged $DPPH^{\cdot}$/g extract)
[d] Total Phenolic Content (mg GAE/g germ)
[e] Antioxidant Activity (mg scavenged $DPPH^{\cdot}$/g germ)

Therefore, if an oil extraction with antioxidant properties is aimed, than the optimum condition for extraction yield should be used, optimum conditions for TPC or AA of the extracts should be used for high antioxidative extracts. If high AA of the extract is aimed, than the latter case will be more economically liable, since a high value product is obtained at low pressures (i.e. with low capital cost). Table 3.15 shows optimum values for parameters obtained by multiple response optimization at their coded values.

Table 3.15 Coded Optimum Values for Factors (Multiple Response Optimization for SC-CO_2 Extraction)

Factor	Coded Value
X_1	-0.19585
X_2	1.60207
X_3	-1.68179

Optimum conditions were 349 bar, 60ºC, 10 min (Table 3.16). At these conditions the optimum yield was 5.6% while TPC and AA of the extracts were 6 mg GAE/g extract and 54.3 mg Scavenged $DPPH^{\cdot}$/g extract, respectively (Table 3.17).

Table 3.16 Uncoded Optimum Values for Factors (Multiple Response Optimization for SC-CO_2 Extraction)

Factor	Uncoded Value
Pressure (bar)	349 bar
Temperature (ºC)	60 ºC
Time (min)	10 min

Table 3.17 Optimum Values of Responses (Multiple Response Optimization for Supercritical CO_2 Extraction)

Response	Optimum Value
Yield[a]	5.6
TPC[b]	6
AA[c]	54.3

[a] % (g extract ×100/g germ)
[b] mg GAE/g extract)
[c] mg scavenged $DPPH^{\cdot}$/g extract

3.4 COMPARISON OF UAE AND SC-CO_2 EXTRACTION

Optimized parameters for UAE were ultrasonication time (1-11 min), temperature (20-60ºC), and ethanol level (5-95%). Pressure (148-602 bar), temperature (40-60ºC) and time (10-60 min) were parameters for SC-CO_2 extraction).

Within this range extraction yield obtained by UAE was between 15-53% (Table 3.4) where those obtained by SC-CO_2 extraction was between 3-9% (Table 5.10). The reason of the low extraction yields obtained by SC-CO_2 extraction was that only oil in the wheat germ was soluble in SC-CO_2. 9% extraction yield showed that almost 100% oil recovery was achieved at those conditions. However, by UAE not only oil, but also phospholipids, waxes, proteins and carbohydrates were extracted.

TPC range (3-7 mg GAE/g extract) of SC-CO_2 extracts was very low compared to TPC of the UAE extracts (13-22 mg GAE/ g extract). Because the solvent (aqueous ethanol) used in UAE is more polar than SC-CO_2, more phenolic compounds were extracted by UAE.

AA of the extracts obtained by UAE ranged between 31-60 mg scavenged $DPPH^{\cdot}$/g extract. AA of the extracts obtained by low ethanol levels (5-35%) were comparable with the AA of those (40-165 mg scavenged $DPPH^{\cdot}$ /g extract) obtained by SC-CO_2 extraction. Extracts obtained by SC-CO_2 were richer in tocopherol and this increased the AA of these extracts made them comparable with the extracts obtained by UAE which were rich in phenolics.

Tocopherols as being fat soluble, were expected to be highly extracted by SC-CO_2 extraction rather than UAE. Bruni et al. (2002) compared tocopherol contents of the Amaranthus Caudatus (A.C) seed extracts obtained by UAE (1 hour, 25ºC and 5 g seed/100 ml methanol) and SC-CO_2 (202.65&405 atm, 15

min, 40ºC). It was reported that higher amount of tocopherol (0.129 mg/g A.C seed) was extracted by SC-CO_2 extraction than UAE (0.064 mg tocopherol/g A.C seed).

However, in present study TTC of the extracts calculated on "per g germ" basis (Table 3.13) indicated that almost same amount of tocopherol was extracted by both of ultrasound assisted extraction (at optimum conditions for AA of the extracts: 0.33 mg total tocopherol/g germ) and SC-CO_2 (0.31 mg total tocopherol/ g germ). Therefore, shorter ultrasonication times might increase the yield of tocopherol by UAE compared to SC-CO_2 extraction.

Yield obtained by multiple response optimization of UAE was 31.10% which was six times higher than the 5.6% yield obtained by SC-CO_2 extraction (Table 3.18). Also TPC and AA of the extracts obtained by UAE were higher than the ones by SC-CO_2 Extractions.

Table 3.18 Comparison of Extraction Yield, and TPC, AA and TTC of The Extracts Obtained by UAE and SC-CO_2 Extractions at Optimum Conditions

Extraction	Yield[a]	TPC[b]	AA[c]	TTC[d]
UAE	31.10	19.3	135.8	1.170 [e]
SC-CO_2 Extraction	5.6	6	54.3	7.142 [f]

[a] % (g extract/g germ×100)
[b] Total Phenolic Content (mg GAE/g extract)
[c] Antioxidant Activity (mg scavenged $DPPH^{\cdot}$/g extract)
[d] Total Phenolic Content (mg total tocopherol/g extract)
[e] U.time=9 min, T=58°C, ethanol level=95% (conditions of max. AA)
[f] P=240 bar, T=56°C, t=20 min (conditions of max TTC)

CHAPTER 4

CONCLUSION

In this study extraction of antioxidants from wheat germ was aimed. Two different modern solvent extraction techniques ultrasound assisted" and supercritical CO_2 extraction were experimented. The extracts obtained by UAE had higher TPC (mg GAE/g extract) and AA (mg scavenged $DPPH^{\cdot}$/g extract) than the extracts obtained by SC-CO_2 extraction. However TTC (mg total tocopherol/g extract) of the UAE extracts were much lower than the other.

Extraction conditions can be selected whether the amount (yield) or the contents of the extracts is more important.

UAE (at ~60°C) with low ethanol level (~5-30%) and short times (1-3 min) provided protein rich extracts with high yield, with medium TPC and AA. Those extracts especially could be used in lotions where the total amount of antioxidants is not important. Also, they could be used as nutritional supplements since they are rich both in water soluble proteins and carbohydrates. On the other hand, with high ethanol level (~90%) and long times (6-11 min), waxy structured extracts with low yield but high TPC and AA were obtained. These extracts could be used in cosmetic products (soap, lotions and perfumes), health supplements and chemicals. High phospholipids and waxes will be a benefit in soap production and cosmetics.

Extracts of SC-CO_2 extraction had higher tocopherol and oil content than those of UAE. Those could be more valuable in food additives since there is no risk of contamination with an organic solvent. Extracts obtained by short time (~10 min) SC-CO_2 extraction at low pressures (~150 bar) yielded low oil content and also

had high TPC and AA. Those characteristics of the extracts would be advantageous for nutritional supplement production. However, extracts obtained at high pressures (~ 450 bar) was rich in oil content but less concentrated in TTC (mg tocopherol/ g extract). Those extracts could be preferred as oil additives. SC-CO_2 defatted wheat germ has potential uses as a rich protein and mineral source, as well.

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APPENDIX A

PROCEDURES

A.1 SPOUTED AIR BED STABILIZATION of WHEAT GERM

The dryer was constructed using a 250 m^3/ h fan, a 3.2 kW electrical heater, a cylindrical gas column with a length of 1.5 m and a diameter of 16 cm. The column had an inverse conical base of 6.2 cm inlet diameter at the cone bottom. 330 g of wheat germ is used for each run. Spouted bed was operated at 216 ºC with an air flow rate 55 m^3/ h for 7 min. Figure A.1 is a schematic of spouted bed.

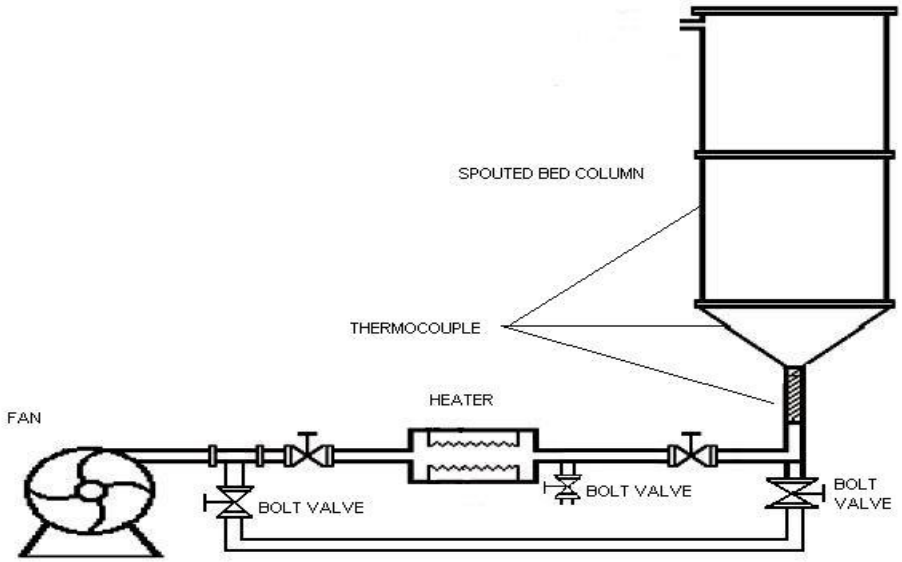

Figure A.1 Spouted Bed (Oymak, 2006)

A.2 SIEVE ANALYSIS

The procedure used in sieve analysis of roasted wheat germ and milled-roasted wheat germ was:

i. Each sieve and the pan was weighed and recorded, w_{pi}.

ii. Sieves were placed in a mechanical sieve shaker. They were placed in an increasing sieve number or in a decreasing size of openings. Coarsest sieve was at the top and finest was just at the bottom just above the pan.

iii. Approximately 200 grams of roasted wheat germ or milled-roasted wheat germ was weighed, w_s.

iv. The prepared sample was poured into the top of the sieves, and the cover was placed tightly on top.

v. Shaker was operated for 10 minutes.

vi. Each sieve was weighed with its contents, w_p.

Percent of retained sample on each sieve was calculated by equation A.1.

$$\text{Retained sample, } \% = \frac{w_{pi} - w_p}{w_s} \cdot 100 \qquad \text{(A.1)}$$

w_{pi}: weight of the empty sieve or pan, g

w_s: weight of the sample, g

w_p: weight of the sieve after 10 minutes shaking, g

For roasted wheat germ sieve mesh number 14, 16, 18, 20, 25, 30 and 35 were used. Sieve mesh number 35, 50, 60, 70, 80, 100, 120 and 140 were used for milled- roasted wheat germ. Particle size conversion chart was given in Table-A.1.

Table A.1: Particle Size Conversion Chart (Sigma-Aldrich, 2008)

Sieve Designation		Nominal Sieve Opening		
Standard	***Mesh***	***inches***	***mm***	***Microns***
2.00 mm	No. 10	0.0787	2.00	2000
1.68 mm	No. 12	0.0661	1.68	1680
1.41 mm	**No. 14**	**0.0555**	**1.41**	**1410**
1.19 mm	**No. 16**	**0.0469**	**1.19**	**1190**
1.00 mm	**No. 18**	**0.0394**	**1.00**	**1000**
841 µm	**No. 20**	**0.0331**	**0.841**	**841**
707 µm	**No. 25**	**0.0278**	**0.707**	**707**
595 µm	**No. 30**	**0.0234**	**0.595**	**595**
500 µm	**No. 35**	**0.0197**	**0.500**	**500**
420 µm	No. 40	0.0165	0.420	420
354 µm	No. 45	0.0139	0.354	354
297 µm	**No. 50**	**0.0117**	**0.297**	**297**
250 µm	**No. 60**	**0.0098**	**0.250**	**250**
210 µm	**No. 70**	**0.0083**	**0.210**	**210**
177 µm	**No. 80**	**0.0070**	**0.177**	**177**
149 µm	**No. 100**	**0.0059**	**0.149**	**149**
125 µm	**No. 120**	**0.0049**	**0.125**	**125**
105µm	**No. 140**	**0.0041**	**0.105**	**105**
88 µm	No. 170	0.0035	0.088	88
74 µm	No. 200	0.0029	0.074	74
63 µm	No. 230	0.0025	0.063	63
53 µm	No. 270	0.0021	0.053	53

A.3 MOISTURE CONTENT ANALYSIS

The moisture content of the raw and roasted wheat germ was determined according to AACC Method 44-20 (AACC, 2000). The procedure was:

i. A glass dish and its lid are dried in the oven to a constant weight.

ii. The lid and dish were cooled in a dessicator and together the weight was recorded as w_1.

iii. Accurately 5 grams of sample was weighed into predried dish and dish containing the sample was weighed, w_g.

iv. The glass dish was put in an oven maintained at 105 °C and dried for four hours.

v. After for four hours the dish was covered with the lid and placed into desiccator to cool to the room temperature.

vi. The glass dish and its content was weighed, w_2.

The moisture content was calculated on the wet basis of the raw and roasted wheat germ using Equation A.2.

$$\text{Moisture Content, } \% = \frac{w_g - \left(w_2 - w_1\right)}{w_g} \cdot 100 \qquad \text{(A. 2)}$$

w_g: weight of the sample wet basis, g

w_1: weight of the empty glass dish, g

w_2: weight of the glass dish and its content, g

Experiments were done in triplicates and arithmetic mean of results was given.

A.4 CRUDE FAT ANALYSIS

Fat analysis of roasted and raw wheat germ was done by direct extraction with petroleum ether (boiling range 40-60 °C) by Soxhlet extractor according to AACC Method 30-25 (AACC, 2000).

i. Raw wheat germ was predried at 105°C in oven for 4 hours to easy the penetration and cooled in dessicator.

ii. Soxhlet cellulose thimble was dried in oven and cooled in dessicator.

iii. 5 g sample was weighed, w_{fs}, and transferred into extraction thimble. The end of the thimble was plugged with cotton. The thimble was placed in the central siphon portion of the Soxhlet extractor.

iv. 250 mL ground glass joint flask was dried in oven, cooled in dessicator and weighed, w_{f1}. Petroleum ether about 100 mL was put into the flask. Finally the flask was placed on the heater and connected to Soxhlet siphon and condenser.

v. Reflux for 5 hours at condensation rate of 5-6 drops/sec.

vi. After extraction, ether was removed from flask at low temperature (below auto ignition temperature of solvent used) volatilization before oven drying. (AACC)

vii. Fat remaining flask was dried in oven at 105°C for 30 minutes. The weight of the flask was recorded, w_{f2}.

The experiments were studied in triplicates and calculations are done on wet basis of the raw wheat germ by following formula:

$$\text{Fat, \%} = \frac{w_{f2} - w_{f1}}{w_{fs}} \qquad \text{(A. 3)}$$

A.5 ASH CONTENT ANALYSIS

Ash content of raw and roasted wheat germ was determined according to the AOAC Method 923.03 (AOAC, 1995). Dry-ashing procedure had the following steps:

i. Porcelain crucibles were ignited, cooled and tared, w_{cb}.

ii. 5 g sample was weighed into the each crucible, w_{as}.

iii. Crucibles were placed in muffle furnace preheated to 550 °C.

iv. Crucibles were hold at 550 °C since light gray or white ash was observed (or overnight).

v. Crucibles were transferred into a dessicator, covered with their lids and cooled to room temperature.

vi. Final weights of crucibles were recorded, w_{ca}.

The ash content was calculated on the wet basis of raw and roasted wheat germ by Equation. A. 4.

$$\text{Ash Content, } \% = \frac{w_{cb} - w_{ca}}{w_{as}} \cdot 100 \qquad \text{(A. 4)}$$

w_{cb}: weight of empty crucibles before ashing, g

w_{as}: weight of ashing sample, g

w_{ca}: weight of crucibles and its contents after ashing, g

A.6. TOTAL PHENOLIC CONTENT

Folin- Ciocalteu assay was used to determine the total phenol content of roasted wheat germ extracts. Procedures, followed after Solid-Liquid Separation were stated below.

GALLIC ACID STOCK SOLUTION

0.1 g dry Gallic acid was dissolved in 1 mL of ethanol and diluted to a volume 10 mL with deionised water. Stock solution was 10 g Gallic acid/L.

SODIUM CARBONATE SOLUTION

40 g of anhydrous sodium carbonate was dissolved in 160 mL of deionized water and heated on magnetic stirrer until boils. The solution was cooled to room temperature and a few sodium crystals were added. After keeping

the solution for 24 hours, volume was made up to 200 mL with deionized water and it was filtered. Again volume was made up to 200 mL to get a final 20% solution.

GALLIC ACID CALIBRATION CURVE

A calibration curve of gallic acid was prepared to determine the levels in the samples. Results were reported at Gallic Acid Equivalent (GAE). 1 mL of Gallic acid stock solution was made up to a volume of 10 ml with deionized water. Final solution concentration was 1000 mg Gallic acid/L. 0, 0.5, 1, 1.5, 2, 2.5 3, 4, 5mL of the 1000 mg Gallic acid/L Gallic acid solution were diluted to 10 mL. Final solutions were 0, 50, 100,150,200, 250, 300, 400 and 500 mg Gallic acid/L.

Absorbance data for different Gallic acid solutions are given below;

Table A.2 Absorbance Data for Gallic Acid Calibration Curve

GALLIC ACID mg/L	**ABSORBANCE 766,2nm**
50	0,0693
100	0,1317
150	0,1945
200	0,2715
250	0,3116
300	0,4074
400	0,5348
500	0,6526

Calibration curve procedure;

i. 60 µL of each solution was taken and put into the test tube (for blank solution 60 µL deionised water was used.)
ii. 4, 74 mL of deionised water was added to tubes and vortexed.

iii. 300 µL of non-diluted Folin- Ciocalteu reagent was added and vortexed. The solutions were kept in dark at room conditions.

iv. 900 µL of 20 % sodium carbonate solution was added and vortexed.

v. Test tubes were placed into shaking water bath at 40 °C and kept for 30 minutes.

vi. Solutions were read against blank at 766, 2 nm.

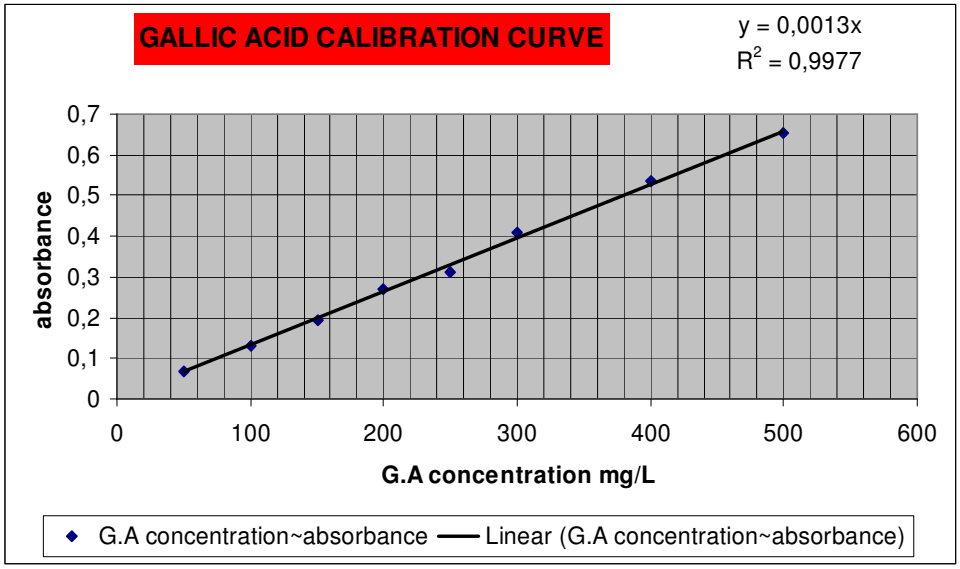

Figure A.2 Gallic Acid Calibration Curve

Analysis procedure for extracts;

i. 60 µL of each extract was taken and put into the test tube (for blank solution 60 µL deionized water was used.)

ii. 4, 74 mL of deionized water was added to tubes and vortexed.

iii. 300 µL of non-diluted Folin-Ciocalteu reagent was added and vortexed. The solutions were kept in dark at room conditions.

iv. 900 μL of 20 % sodium carbonate solution was added and vortexed.

v. Test tubes were placed into shaking water bath at 40 °C and kept for 30 minutes.

vi. Solutions were read against blank at 766, 2 nm.

In spectrophotometer analysis wavelength check was done between 755 nm and 780 nm to determine whether 765 nm has the maximum absorbance or not as it is stated in the literature. 766, 2 nm was recorded to have maximum absorbance.

A.7 TOTAL ANTIOXIDANT ACTIVITY

Free radical scavenging capacity of roasted wheat germ extracts was determined using the 2, 2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$). The solution of $DPPH^{\cdot}$ in ethanol (6*10-5 M) was freshly made before UV measurements. 2,925 ml of this solution was mixed well with 0,075 ml of extract and the absorbance was measured at 515, 1 nm against a blank of pure ethanol at 60 min as the reaction reached plateau. The $DPPH^{\cdot}$ concentration in the reaction medium was calculated from the following calibration curve:

$$A_{515,\,1\,nm} = 32,47\,[DPPH^{\cdot}]_t \qquad (A.5)$$

According to the curve the percent inhibition of the $DPPH^{\cdot}$ levels were estimated by using the following formula:

$$\%Inhibition = [([DPPH^{\cdot}]_i - [DPPH^{\cdot}]_t) / [DPPH^{\cdot}]_i] / 100 \qquad (A.6)$$

Where $[DPPH^{\cdot}]_i$ was the absorbance of the $DPPH^{\cdot}$ concentration at t= 0 min and $[DPPH^{\cdot}]_t$ was the absorbance of the $DPPH^{\cdot}$ concentration at t=60 min. Calibration curve was prepared by different final $[DPPH^{\cdot}]$ concentrations for 3 mL of extract-$[DPPH^{\cdot}]$ mixture. The concentration used for the experiments ($23.66*10^{-3}$ g $[DPPH^{\cdot}]$/L) was assumed to be 100 %.

In order to get absorbance results in the linear range for spectrophotometer

reading, instead of 75 µl extract, 10 µL sample was diluted with 65 µl pure ethanol for ultrasonication and 20 Ml

sample was diluted with for 55 µl supercritical CO_2 . In spectrophotometer analysis wavelength check was done between 510 nm and 520 nm to determine if 515 nm (stated by the literature) had the maximum absorbance or not. 515.1 nm was recorded to have maximum absorbance.

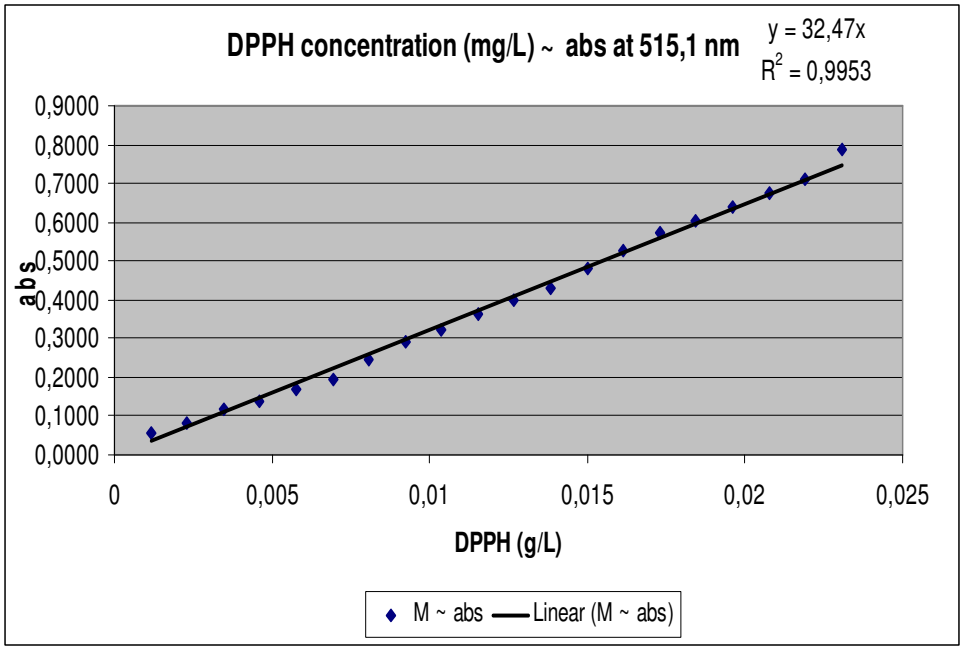

Figure A.3 [DPPH˙] Concentration Calibration Curve

Table A.3 Absorbance Data for [DPPH˙] Calibration Curve

DPPH˙ percentage %	**DPPH˙ concentration (g/L)**	**Absorbance at 515,1 nm**
100	0,02365	0,7877
95	0,02191	0,7097
90	0,02076	0,6765
85	0,01960	0,6381
80	0,01845	0,6046
75	0,01730	0,5749
70	0,01615	0,5261
65	0,01499	0,4796
60	0,01384	0,4313
55	0,01268	0,3992
50	0,01153	0,3636
45	0,01038	0,3235
40	0,00923	0,2934
35	0,00807	0,2473
30	0,00692	0,1954
25	0,00576	0,1674
20	0,00461	0,1381
15	0,00346	0,1176
10	0,00231	0,0793
5	0,00115	0,0569

APPENDIX B

EXPERIMENTAL DATA

B.1 ULTRASOUND ASSISTED EXTRACTION DATA

Table B.1 Experimental Yield for UAE

Exp. no.	g extract/ 100 mL sample	g extract/ g wheat germ	Yield[a]
1	1.65	0.330	33.0
2	1.63	0.326	32.6
3	2.56	0.512	51.2
4	0.88	0.176	17.6
5	1.82	0.364	36.4
6	2.19	0.438	43.8
7	0.86	0.173	17.3
8	2.69	0.538	53.8
9	1.13	0.226	22.6
10	1.72	0.344	34.4
11	2.55	0.510	51.0
12	0.78	0.156	15.6
13	1.77	0.354	35.4
14	2.01	0.402	40.2
15[b]	1.70	0.340	34.0
16	1.86	0.372	37.2
17	1.77	0.354	35.4
18	2.00	0.400	40.0
19	1.82	0.364	36.4
20	2.00	0.400	40.0
21	1.95	0.390	39.0
22	1.87	0.374	37.4
23	1.79	0.358	35.8

[a] % (g extract ×100/g germ)

[b] Experiments 15-23 are replications at center point conditions.

Table B.2 Absorbance Data for TPC of UAE

Exp. no.	TPC abs	mg GAE/L	TPC[a]	TPC[b]
1	0.3509	269.92	16.4	5.4
2	0.3750	288.46	17.7	5.8
3	0.4060	312.31	12.2	6.2
4	0.1840	141.54	16.1	2.8
5	0.3222	247.85	13.6	5.0
6	0.4381	337.00	15.4	6.7
7	0.2441	187.77	21.8	3.8
8	0.4470	343.85	12.8	6.9
9	0.2340	180.00	15.9	3.6
10	0.3760	289.23	16.8	5.8
11	0.4111	316.23	12.4	6.3
12	0.1570	120.77	15.4	2.4
13	0.3460	266.15	15.0	5.3
14	0.4680	360.00	17.9	7.2
15[c]	0.3875	298.08	17.5	6.0
16	0.3846	295.85	15.9	5.9
17	0.3985	306.54	17.3	6.1
18	0.3826	294.31	14.7	5.9
19	0.3611	277.77	15.3	5.6
20	0.3944	303.38	15.2	6.1
21	0.3920	301.54	15.5	6.0
22	0.3411	262.38	14.0	5.2
23	0.3933	302.54	16.9	6.1

[a] Total Phenolic Content (mg GAE/g extract).
[b] Total Phenolic Content (mg GAE/g germ).
[c] Experiments 15-23 are replications at center point conditions.

Table B.3 Absorbance Data for AA of UAE

Exp. no.	blank abs	AA abs	mg scavenged DPPH· /L	AA[a]	AA[b]
1	0.684	0.5616	3.77	68.6	22.6
2	0.949	0.6584	8.98	165.3	53.9
3	0.628	0.5158	3.48	40.7	20.9
4	0.659	0.5669	2.85	97.2	17.1
5	0.905	0.7424	5.02	82.8	30.1
6	0.717	0.5262	5.88	80.6	35.3
7	0.722	0.6395	2.55	88.7	15.3
8	0.903	0.7426	4.95	55.3	29.7
9	0.736	0.6383	3.00	79.8	18.0
10	0.724	0.5526	5.28	92.1	31.7
11	0.621	0.4726	4.58	53.9	27.5
12	0.724	0.6181	3.27	125.9	19.6
13	0.707	0.5437	5.06	85.8	30.4
14	0.701	0.5469	4.77	71.1	28.6
15[c]	0.949	0.6673	8.71	153.6	52.2
16	0.949	0.6755	8.45	136.5	50.7
17	0.722	0.5298	5.94	100.7	35.7
18	0.968	0.6872	8.67	130.0	52.0
19	0.956	0.6899	8.23	135.7	49.4
20	0.934	0.6789	7.90	118.4	47.4
21	0.755	0.5406	6.61	101.8	39.7
22	0.965	0.6998	8.19	131.3	49.1
23	0.723	0.5170	6.38	106.9	38.3

[a] Antioxidant Activity (mg scavenged DPPH·/g extract)
[b] Antioxidant Activity (mg scavenged DPPH·/g germ)
[c] Exp no. 15-23 are replications at center point conditions.

B.2 SUPERCRITICAL CO_2 EXTRACTION

Table B.4 Experimental Yield for SC-CO_2 Extraction

Exp. no.	Wheat Germ (g)	Extract (g)	Yield[a]
1	2.0008	0.164	8.20
2	2.0001	0.169	8.43
3	2.0005	0.163	8.17
4	2.000	0.179	8.95
5	2.0005	0.163	8.15
6	2.0009	0.17	8.50
7	2.0002	0.17	8.51
8	2.0004	0.124	6.21
9	2.0001	0.16	8.01
10	2.0004	0.148	7.39
11	2.0007	0.08	3.98
12	2.0001	0.145	7.27
13	2.0004	0.081	4.04
14	2.0000	0.055	2.76
15[b]	2.0004	0.174	8.68
16	2.0006	0.173	8.64
17	2.0003	0.169	8.44
18	2.0000	0.167	8.33
19	2.0008	0.166	8.32
20	2.0005	0.165	8.26
21	2.0007	0.164	8.19
22	2.0009	0.162	8.11
23	2.0009	0.16	8.02

[a] % (g extract ×100/g germ)
[b] Exp no. 15-23 are replications at center point conditions.

Table B.5 Absorbance Data for TPC of SC-CO_2 Extraction

Exp. no.	TPC abs	mg GAE/L	TPC[a]	TPC[b]
1	0.1398	107.54	2.619	0.2
2	0.2470	190.00	4.507	0.4
3	0.2115	162.69	3.982	0.3
4	0.2268	174.46	3.899	0.4
5	0.1709	131.46	3.225	0.3
6	0.2613	201.00	4.727	0.4
7	0.1864	143.38	3.369	0.3
8	0.1572	120.92	3.894	0.2
9	0.1460	112.30	2.804	0.2
10	0.2122	163.23	4.417	0.3
11	0.1716	132.00	6.631	0.3
12	0.1765	135.77	3.735	0.3
13	0.1684	129.54	6.412	0.3
14	0.0975	75.00	5.435	0.2
15[c]	0.2243	172.54	4.301	0.3
16	0.2179	167.62	4.024	0.3
17	0.2103	161.77	3.916	0.3
18	0.2087	160.54	3.698	0.3
19	0.1990	153.08	3.737	0.3
20	0.1986	152.77	3.671	0.3
21	0.1963	151.00	3.578	0.3
22	0.1884	144.92	3.354	0.3
23	0.1859	143.00	3.525	0.3

[a] Total Phenolic Content (mg GAE/g extract).
[b] Total Phenolic Content (mg GAE/g germ).
[c] Exp no. 15-23 are replications at center point conditions.

Table B.6 Absorbance Data for AA Data of SC-CO_2 Extraction

Exp. no.	blank abs	AA abs	mg scavenged $DPPH^{\cdot}$ /L	AA[c]	AA[e]
1	0.6788	0.517	4.99	18.228	7.1
2	0.6788	0.482	6.09	21.686	8.7
3	0.6567	0.404	7.81	28.686	11.6
4	0.6788	0.379	9.28	31.108	13.3
5	0.6789	0.399	8.65	31.840	12.4
6	0.7088	0.482	7.02	24.766	9.6
7	0.6787	0.347	10.27	36.193	14.7
8	0.903	0.7426	4.95	56.481	13.9
9	0.736	0.6383	3.00	37.934	14.5
10	0.724	0.5526	5.28	26.404	10.6
11	0.621	0.4726	4.58	54.634	11.1
12	0.724	0.6181	3.27	39.860	13.8
13	0.707	0.5437	5.06	59.388	12.0
14	0.701	0.5469	4.77	52.888	13.9
15[f]	0.6090	0.334	8.50	31.765	13.5
16	0.6720	0.367	9.44	34.000	13.6
17	0.6901	0.354	10.38	37.706	14.6
18	0.6181	0.348	8.34	28.812	13.1
19	0.6898	0.372	9.81	35.913	13.8
20	0.7324	0.361	11.49	41.428	15.2
21	0.6970	0.37	10.10	35.898	14.1
22	0.6789	0.355	10.02	34.795	14.3
23	0.7332	0.383	10.84	40.071	14.3

[a] Antioxidant Activity (mg scavenged $DPPH^{\cdot}$/g extract)
[b] Antioxidant Activity (mg scavenged $DPPH^{\cdot}$/g germ)
[c] Exp no. 15-23 are replications at center point conditions.

Table B.7 TTC of Extracts for SC-CO_2 Extraction

Exp. no.	δ- mg/mL	β- & γ- mg/mL	α- mg/mL	Total Tocop. mg /ml	TTC[a]	TTC[b]
1		0.036	0.0781297	0.114	0.229	2.784
2		0.042	0.0920926	0.135	0.269	3.192
3		0.048	0.1067384	0.154	0.309	3.78
4		0.045	0.0875622	0.133	0.265	2.961
5		0.042	0.0901672	0.133	0.265	3.252
6		0.041	0.0980779	0.139	0.279	3.28
7	1E-05	0.036	0.0833796	0.12	0.239	2.81
8		0.047	0.1025619	0.149	0.298	4.806
9		0.046	0.0957239	0.142	0.283	3.537
10		0.028	0.0703697	0.098	0.196	2.648
11	2E-05	0.047	0.0948786	0.142	0.284	7.142
12		0.036	0.0760117	0.112	0.224	3.08
13	2E-05	0.047	0.0957346	0.142	0.284	7.04
14		0.017	0.036162	0.053	0.106	3.845
15[c]		0.046	0.1020978	0.148	0.296	3.69
16	4E-05	0.044	0.0987589	0.143	0.286	3.428
17	2E-05	0.048	0.10315	0.151	0.302	3.653
18		0.049	0.1050943	0.154	0.309	3.556
19	3E-05	0.049	0.10255	0.151	0.303	3.696
20	3E-05	0.045	0.09475	0.14	0.279	3.354
21		0.046	0.0979128	0.144	0.289	3.42
22	4E-05	0.048	0.0971981	0.145	0.291	3.367
23		0.047	0.0991	0.147	0.293	3.612

[a] Total Tocopherol Content (mg tocopherol/g germ)
[b] Total Tocopherol Content (mg tocopherol/g extract)
[c] Experiments 15-23 are replications at center point conditions.

APPENDIX C

STATISTICAL CALCULATIONS

The F-ratio provides a statistic that can be compared to a probability distribution table for a given confidence level to determine whether the treatment means are significantly different. Generally two levels of confidence are used 95% and 99%. There are an infinite number of F-distributions based upon confidence levels, degrees of freedom for factors, and degrees of freedom for the unexplained variation.

The F-ratio is equivalent to the Mean Square (variation) between the groups divided by the Mean Square error within the groups. Also, degrees of freedom (Df) for the numerator and the denominator of the F-ratio are needed in order to select a critical F-value from the statistical table (Nist, 2007). If the calculated F-ratio for the model is higher than the one obtained from the statistical table (Figure C.1), a significant relationship exists between response and independent variables.

At 95% confidence level and present Df values (numerator: 9, denominator: 13), obtained critical F-ratio was 2.71. Analysis of variance (ANOVA) was performed for model of each response.

d/n	1	2	3	4	5	6	7	8	9	10
1	161.45	199.50	215.71	224.58	230.16	233.99	236.77	238.88	240.54	241.88
2	18.51	19.00	19.16	19.25	19.30	19.33	19.35	19.37	19.39	19.40
3	10.13	9.55	9.28	9.12	9.01	8.94	8.89	8.85	8.81	8.79
4	7.71	6.94	6.59	6.39	6.26	6.16	6.09	6.04	6.00	5.96
5	6.61	5.79	5.41	5.19	5.05	4.95	4.88	4.82	4.77	4.74
6	5.99	5.14	4.76	4.53	4.39	4.28	4.21	4.15	4.10	4.06
7	5.59	4.74	4.35	4.12	3.97	3.87	3.79	3.73	3.68	3.64
8	5.32	4.46	4.07	3.84	3.69	3.58	3.50	3.44	3.39	3.35
9	5.12	4.26	3.86	3.63	3.48	3.37	3.29	3.23	3.18	3.14
10	4.97	4.10	3.71	3.48	3.33	3.22	3.14	3.07	3.02	2.98
11	4.84	3.98	3.59	3.36	3.20	3.10	3.01	2.95	2.90	2.85
12	4.75	3.89	3.49	3.26	3.11	3.00	2.91	2.85	2.80	2.75
13	4.67	3.81	3.41	3.18	3.03	2.92	2.83	2.77	2.71	2.67
14	4.60	3.74	3.34	3.11	2.96	2.85	2.76	2.70	2.65	2.60
15	4.54	3.68	3.29	3.06	2.90	2.79	2.71	2.64	2.59	2.54
16	4.49	3.63	3.24	3.01	2.85	2.74	2.66	2.59	2.54	2.49
17	4.45	3.59	3.20	2.97	2.81	2.70	2.61	2.55	2.49	2.45
18	4.41	3.56	3.16	2.93	2.77	2.66	2.58	2.51	2.46	2.41
19	4.38	3.52	3.13	2.90	2.74	2.63	2.54	2.48	2.42	2.38
20	4.35	3.49	3.10	2.87	2.71	2.60	2.51	2.45	2.39	2.35

Figure C.1 Upper Critical Values of the F Distribution at 5% Significance Level (n: numerator, d: denominator)

C.1 ULTRASOUND ASSISTED EXTRACTION STATISTICS

The Model F-ratio (Table C.1) for "Extraction Yield %" was found to be 41.49 and implied that the model was significant, since so far exceeded the critical F-ratio (2.71).

Table C.1 ANOVA of "Extraction Yield (%)" for UAE

Source	Sum of Squares	Df	Mean Square	F-Ratio	p-Value
Model	2132.33	9	236.925	41.49	0.0000
Residual	74.2355	13	5.71043		
Total (Corr.)	2206.56	22			

R-squared = 96.6357 percent

Using a 95% confidence level, the F-value of "TPC" was 3.72 (Table C.2). Since the F-ratio of 3.72 exceeded 2.71, we can conclude that there was less than a 5% probability that the difference in means was due to random chance.

Table C.2 ANOVA of "TPC (mg GAE/g extract)" for UAE

Source	Sum of Squares	Df	Mean Square	F-Ratio	p-Value
Model	68.991	9	7.66566	3.72	0.0161
Residual	26.7999	13	2.06153		
Total (Corr.)	95.7909	22			

R-squared = 72.0225 percent

F-ratio (3.53) for "AA" of UAE extracts implied that the model was significant and there was only a 1.98% probability that a Model F-ratio this large could occur (Table C.3).

Table C.3 ANOVA of "AA (mg scavenged DPPH˙/g extract)" for UAE

Source	Sum of Squares	Df	Mean Square	F-Ratio	p-Value
Model	16762.1	9	1862.46	3.53	0.0198
Residual	6864.75	13	528.058		
Total (Corr.)	23626.9	22			

R-squared = 70.9452 percent

Table C.4 Replication Points (Exp no. 15-23) Statistics for UAE

	Yield[a]	TPC[b]	AA[c]	TPC[d]	AA[e]
Mean	37.2	15.8	123.9	5.9	46.1
Standard Error	0.7	0.4	6.0	0.1	2.1
Median	37.2	15.5	130.0	6.0	49.1
Mode	40	#	#	#	#
Standard Deviation	2.1	1.2	18.1	0.3	6.4
Sample Variance	4.4	1.5	327.6	0.1	40.9
Kurtosis	-1.0	-1.1	-1.0	2.0	-1.3
Skewness	0.1	0.2	0.1	-1.6	-0.8
Range	6	3.5	52.9	0.9	16.6
Minimum	34	14.0	100.7	5.2	35.7
Maximum	40	17.5	153.6	6.1	52.2
Sum	335.162	142.3	1115.0	52.8	414.5
Count	9	9	9	9	9.0
Confidence Level (95.0%)	1.6	0.9	13.9	0.2	4.9

[a] % (g extract ×100/g germ)
[b] Total Phenolic Content (mg GAE/g extract)
[c] Antioxidant Activity (mg scavenged DPPH˙/g extract)
[d] Total Phenolic Content (mg GAE/g germ)
[e] Antioxidant Activity (mg scavenged DPPH˙/g germ)

Table C.5 Experimental and Predicted Values of Responses for UAE

	Yield[a]		TPC[b]		AA[c]	
Exp. no.	**Observed Value**	**Fitted Value**	**Observed Value**	**Fitted Value**	**Observed Value**	**Fitted Value**
1	33.00	34.88	16.36	15.77	68.61	85.26
2	32.60	30.91	12.20	12.18	40.74	53.47
3	51.20	50.11	17.70	18.80	165.29	135.76
4	17.60	16.58	16.08	16.96	97.24	91.40
5	36.40	37.49	13.62	13.85	82.78	63.19
6	43.80	45.10	15.39	14.38	80.61	87.90
7	17.26	18.48	12.78	12.80	55.25	50.71
8	53.80	51.79	21.76	19.68	88.74	118.11
9	22.60	37.26	17.53	15.85	153.63	123.41
10	34.40	37.26	15.92	15.85	136.47	123.41
11	51.00	37.26	17.32	15.85	100.73	123.41
12	15.60	37.26	14.72	15.85	130.03	123.41
13	35.40	37.26	15.26	15.85	135.71	123.41
14	40.20	37.26	15.17	15.85	118.44	123.41
15[d]	34.00	37.26	15.46	15.85	101.75	123.41
16	37.16	37.26	14.03	15.85	131.33	123.41
17	35.40	37.26	16.90	15.85	106.90	123.41
18	40.00	20.50	15.93	14.88	79.75	97.29
19	36.40	33.87	16.82	18.05	92.12	94.15
20	40.00	52.58	12.40	12.98	53.92	42.20
21	39.00	37.66	15.04	15.40	85.75	97.73
22	37.40	17.25	15.48	16.96	125.88	95.59
23	35.80	37.52	17.91	16.44	71.13	79.31

[a] % (g extract ×100/g germ)
[b] Total Phenolic Content (mg GAE/g extract)
[c] Antioxidant Activity (mg scavenged $DPPH^{\cdot}$/g extract)
[d] 15-23 are replications at center point conditions.

Table C.6 Regression Coefficients and p-values of Response Models for UAE

Term	Coefficient	Yield[a]		TPC[b]		AA[c]	
		Estimate	**p-Value**	**Estimate**	**p-Value**	**Estimate**	**p-Value**
constant	β_0	37.2552*	**0.0000**	15.8515*	**0.0000**	123.414*	**0.0000**
X_1	β_1	-0.7841	0.2468	-0.2011	0.6134	1.76775	0.7807
X_2	β_2	7.3116*	**0.0000**	-0.1470	0.7112	-2.7922	0.6608
X_3	β_3	-9.9036*	**0.0000**	2.04458*	**0.0002**	20.0385*	**0.0067**
X_1^2	β_{11}	-0.3726	0.5450	0.08946	0.8077	-14.541*	**0.0255**
$X_1 \times X_2$	β_{12}	-0.575	0.5081	0.1875	0.7178	11.45	0.1822
$X_1 \times X_3$	β_{13}	-0.125	0.8847	0.3875	0.4589	7.585	0.3675
X_2^2	β_{22}	-1.5747*	**0.0209**	-0.4320	0.2518	-10.896	0.0812
$X_2 \times X_3$	β_{23}	0.425	0.6234	0.8775	0.1075	13.52	0.1200
X_3^2	β_{33}	-0.7509	0.2324	0.13719	0.7094	-13.790*	**0.0326**

[a] % (g extract ×100/g germ)
[b] Total Phenolic Content (mg GAE/g extract)
[c] Antioxidant Activity (mg scavenged $DPPH^{\cdot}$/g extract)

$$Y = \beta_0 + \beta_1 \times X_1 + \beta_2 \times X_2 + \beta_3 \times X_3 + \beta_{11} \times X_1^2 + \beta_{12} \times X_1 \times X_2 + \beta_{13} \times X_1 \times X_3 + \beta_{22} \times X_2^2 + \beta_{23} \times X_2 \times X_3 + \beta_{33} \times X_3^2$$

C.2 SUPERCRITICAL CO_2 EXTRACTION STATISTICS

Using a 95% confidence level, the F-value for "Extraction Yield (%)" of SC-CO_2 Extraction was 81.29 (Table C.7), it was concluded that the model was significant.

Table C.7 ANOVA of "Extraction Yield (%)" for SC-CO_2 Extraction

Source	Sum of Squares	Df	Mean Square	F-Ratio	p-Value
Model	60.6868	9	6.74297	81.29	0.0000
Residual	1.07836	13	0.082951		
Total (Corr.)	61.7651	22			

R-squared = 98.2541 percent

Using a 95% confidence level, the F-value of "TPC" was 7.75 (Table C.8). Since the F-ratio of "TPC" exceeded 2.71, the model was significant.

Table C.8 ANOVA of "TPC (mg GAE/g extract)" for SC-CO_2 Extraction

Source	Sum of Squares	Df	Mean Square	F-Ratio	p-Value
Model	17.9403	9	1.99336	7.75	0.0006
Residual	3.34186	13	0.257066		
Total (Corr.)	21.2821	22			

R-squared = 84.2973 percent

The model of "AA" was found to be significant since the F-value was 24.53 (Table C.9).

Table C.9 ANOVA of "AA (mg scavenged DPPH˙/g extract)" for SC-CO_2 Extraction

Source	Sum of Squares	Df	Mean Square	F-Ratio	p-Value
Model	2425.71	9	269.523	24.53	0.0000
Residual	142.862	13	10.9894		
Total (Corr.)	2568.57	22			

R-squared = 94.4381 percent

Table C.10 Replication Points (Exp no. 15-23) Statistics for SC-CO_2 Extraction

	Yield[a]	TPC[b]	AA[c]	TPC[d]	AA[e]
Mean	8.332222	3.755957	35.59895	14.07031736	0.31263217
Standard Error	0.074513	0.0952	1.304766	0.213062059	0.00665663
Median	8.32	3.698339	35.898173	14.06489027	0.30605288
Mode	#	#	#	#	#
Standard Deviation	0.223538	0.285599	3.914298	0.639186178	0.01996989
Sample Variance	0.049969	0.081567	15.321729	0.40855897	0.00039880
Kurtosis	-0.69725	0.425221	-0.099639	0.119098352	-0.9135915
Skewness	0.400054	0.698637	-0.20516	0.339361437	0.28394611
Range	0.66	0.947184	12.616248	2.135527313	0.05905343
Minimum	8.02	3.353624	28.812226	13.09238152	0.28587136
Maximum	8.68	4.300808	41.428473	15.22790884	0.34492478
Sum	74.99	33.80361	320.39055	126.6328562	2.81368957
Count	9	9	9	9	9
Confidence Level (95.0%)	0.171827	0.219531	3.0087978	0.491321989	0.01535021

[a] % (g extract ×100/g germ)
[b] Total Phenolic Content (mg GAE/g extract)
[c] Antioxidant Activity (mg scavenged DPPH˙/g extract)
[d] Total Phenolic Content (mg GAE/g germ)
[e] Antioxidant Activity (mg scavenged DPPH˙/g germ)

Table C.11 Replication Points Statistics for TTC of SC-CO_2 Extracts

	TTC[a]	TTC[b]
Mean	3.530723	0.294019
Standard Error	0.046495	0.003087
Median	3.555609	0.292968
Mode	#	#
Standard Deviation	0.139485	0.009262
Sample Variance	0.019456	8.58E-05
Kurtosis	-2.01986	-0.54682
Skewness	-0.08023	0.021901
Range	0.341471	0.029538
Minimum	3.354427	0.279088
Maximum	3.695898	0.308627
Sum	31.7765	2.646173
Count	9	9
Confidence Level (95.0%)	0.107218	0.007119

[a] Total Tocopherol Content (mg tocopherol/g germ)
[b] Total Tocopherol Content (mg tocopherol/g extract)

Table C.12 Experimental and Predicted Values of Responses for SC-CO_2 Extraction

	Yield[a]		TPC[b]		AA[c]	
Exp. no.	**Observed Value**	**Fitted Value**	**Observed Value**	**Fitted Value**	**Observed Value**	**Fitted Value**
1	8.21	8.02	2.619	3.203	18.228	19.468
2	8.43	8.55	4.507	4.456	21.686	21.398
3	8.17	8.342	3.982	3.525	28.686	28.258
4	8.95	8.725	3.899	3.456	31.108	31.484
5	8.15	8.34	3.225	2.682	31.84	30.858
6	8.50	8.35	4.727	4.889	24.766	24.166
7	8.51	8.84	3.369	3.514	36.193	34.636
8	6.21	5.99	3.894	4.641	56.481	56.707
9	8.02	8.33	4.301	3.738	31.765	35.624
10	8.33	8.33	4.024	3.738	34.000	35.624
11	8.26	8.33	3.916	3.738	37.706	35.624
12	8.68	8.33	3.698	3.738	28.812	35.624
13	8.19	8.33	3.737	3.738	35.913	35.624
14	8.32	8.33	3.671	3.738	41.428	35.624
15[d]	8.44	8.33	3.578	3.738	35.898	35.624
16	8.64	8.33	3.354	3.738	34.795	35.624
17	8.11	8.33	3.525	3.738	40.071	35.624
18	8.01	8.26	2.804	3.534	37.934	37.203
19	7.39	7.13	4.417	4.329	26.404	28.327
20	3.98	4.13	6.631	6.443	54.634	55.199
21	4.04	3.85	6.412	5.832	59.388	60.617
22	7.27	7.02	3.735	3.561	39.860	41.230
23	2.76	3.05	5.435	5.744	52.888	50.317

[a] % (g extract ×100/g germ)
[b] Total Phenolic Content (mg GAE/g extract)
[c] Antioxidant Activity (mg scavenged $DPPH^{\cdot}$/g extract)
[d] 15-23 are replications at center point conditions.

Table C.13 Regression Coefficients and p-values of Response Models for SC-CO_2 Extraction

	Yield[a]		TPC[b]		AA[c]	
Coefficient	**Estimate**	**p-Value**	**Estimate**	**p-Value**	**Estimate**	**p-Value**
β_0	8.33022	**0.0000**	3.7389	**0.0000**	35.624	**0.0000**
β_1:	1.47807	**0.0000**	-0.75551	**0.0001**	-9.17173	**0.0000**
β_2:	0.028123	0.7240	0.40273	**0.0116**	-3.87585	**0.0008**
β_3:	0.84705	**0.0000**	-0.33499	**0.0297**	-6.56156	**0.0000**
β_{11}:	-0.98737	**0.0000**	0.25959	0.0621	-0.25864	0.7607
β_{12}:	-0.07	0.9132	0.058	0.2116	0.70425	0.0558
β_{13}:	-0.6975	**0.0006**	0.76125	0.3636	5.00325	**0.0009**
β_{22}:	-0.00802	0.5039	0.16713	0.7514	-1.7464	0.5583
β_{23}:	-0.045	**0.0000**	0.03925	**0.0010**	-1.87125	**0.0009**
β_{33}:	-0.32445	0.6658	0.11976	0.8301	3.55232	0.1344

[a] % (g extract ×100/g germ)
[b] Total Phenolic Content (mg GAE/g extract)
[c] Antioxidant Activity (mg scavenged $DPPH^{\cdot}$/g extract)
$Y= \beta_0+ \beta_1\times X_1+ \beta_2\times X_2+ \beta_3\times X_3+ \beta_{11}\times X_1^2+ \beta_{12}\times X_1\times X_2+ \beta_{13}\times X_1\times X_3+ \beta_{22}\times X_2^2+ \beta_{23}\times X_2\times X_3+ \beta_{33}\times X_3^2$