ULTRASOUND ASSISTED EXTRACTION OF LIPIDS AND ANTIOXIDANTS FROM WHEAT GERM

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Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

ULTRASOUND ASSISTED EXTRACTION OF LIPIDS AND ANTIOXIDANTS FROM WHEAT GERM

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The aim of this thesis work was to extract lipids and antioxidants from wheat germ using an ultrasonic bath. Alternative solvents: Ethanol, isopropanol and acetone were used for the extraction purposes and for the fat content determination hexane was used. Alternative solvents dissolve wax, phospholipids and some other proteins because of their polar nature which increased the yield. Since waxes and some proteins can be added to the soaps and creams which were made from carrier oils, obtaining these substances together with the oil in the extract was very useful. For isopropanol a linear relation was found between the extract ratio and ultrasonication time. The highest extract ratios were achieved with ethanol. For 30 minutes of ultrasonication 0.10 g extract / g germ was obtained.

After the extraction in order to enhance the phase separation between the

solid phase (germ and other solids) and the extract phase, combinations of centrifugation, storage in the refrigerator and decantation were tested. The technique involving storage the extracts in the refrigerator for 24 hours and centrifugation at 2800 rpm for 20 minutes gave the second highest yield but the best phase separation.

Ethanol extracts from both roasted and nonroasted wheat germs were characterized in terms of their total polyphenol contents. For non roasted wheat germs total polyphenol contents of 200 mg gallic acid / L solution and for roasted wheat germs an average of 170 mg gallic acid / L solution were obtained for 30 minutes of ultrasonic extraction.

For nonroasted wheat germs weak but significant linear relations were found between total polyphenol contents of the extracts and ultrasonication time.

Keywords: Wheat Germ, Ultrasound Assisted Extraction, Total Polyphenol Content, Lipid, Antioxidant

ÖZ

BUĞDAY RÜŞEYMİNDEN LİPİD VE ANTİOKSİDANLARIN ULTRASON DESTEKLİ ÖZÜTLENMESİ

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Bu tez çalışmasının amacı buğday rüşeyminden lipid ve antioksidanların ultrason destekli özütlenmesidir. Buğday rüşeymi un sanayinin bir yan ürünüdür, yağ ve antioksidan kapasitesi bakımından oldukça zengindir. Bu tez çalışmasının arkasındaki ana motivasyon sebebi de bu yan üründen pazarlanabilir bir ürün elde etmektir. İşlenmemiş buğday rüşeymleri, raf ömürleri çok kısa olmasından ötürü, ısıl işlemden geçirilmiş ve çiğ rüşeymler ile kıyaslamalar yapılmıştır.

Özütleme işlemi için etil alkol, isopropil alkol, aseton gibi alternatif çözücüler ve hekzan kullanılmıştır. Alternatif çözücüler ile elde edilen özütleme oranları oldukça yüksek olmasına rağmen özütler bulanık bir halde elde

edilmiştir. Çözücülerin polar yapıda olmaları, yağların yanında mum ve fosfolipid gibi proteinleri çözmelerine ve özütleme oranının yüksek çıkmasına sebep olmuştur. Taşıyıcı yağlardan yapılan kremlere mum ve fosfolipidlerin eklenebileceği düşünülürse, özütte yağ ile beraber bu maddelerin elde edilmesi faydalı olmuştur. Yapılan istatistiki analizlerde isopropil alkol için başarılı bir doğrusal denklem bulunmuştur. En yüksek özütleme oranı etanol ile elde edilmiştir. 30 dakika ultrasonikasyon ile 0.10 g özüt / g rüşeym özütleme oranı elde edilmiştir.

Özütleme işleminden sonra katı ve sıvı fazların ayrılması detaylı bir şekilde incelenmiştir. Analizlerde dört farklı yöntem test edilmiş ve bunların içinden 24 saat buzdolabında bekletme ve arkasından 20 dakika boyunca 2800 devir/dakika santrifüj işleminin en iyi yöntem olduğu tespit edilmiştir.

Etil alkol özütlerinin toplam polifenol kapasiteleri incelenmiştir; çiğ rüşeymler için ortalama 200 mg gallik asit / L çözelti ve işlenmiş rüşeymler için ortalama 170 mg gallik asit / L çözelti değerlerine 30 dakika ultrasonikasyon ile ulaşılmıştır. Yapılan istatistiki analizlerde çiğ buğdaylar için başarılı doğrusal modeller bulunmuştur.

Anahtar kelimeler: Buğday Rüşeymi, Ultrason Destekli Özütleme, Toplam Polifenol Kapasitesi, Yağ, Antioksidan

To My Parents

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NOMENCLATURE

Latin Capital and Lowercase Letters

$F_{CRITICAL}$: Critical F Ratio

P_C : Critical Pressure

T_C : Critical Temperature

t_S : Time of sonication, min

v_S : Volume of the solvent, ml

w_{B1} : Weight of the empty beaker, g

w_{B2} : Weight of the beaker and its contents, g

w_{C1} : Weight of the empty crucible, g

w_{C2} : Weight of the crucible and its contents, g

w_{G1} : Weight of the empty watch glass, g

w_{G2} : Weight of the watch glass and its contents, g

w_S : Weight of the sample, wet basis, g

***Abbreviations*:**

ASE	: Accelerated Solvent Extraction
AUSAS	: Ankara Un Sanayii Anonim Şirketi
EFA	: Essential Fatty Acids
GA	: Gallic Acid
LOF	: Lack of Fit
MAE	: Microwave Assisted Extraction
SFE	: Supercritical Fluid Extraction
TPC	: Total Polyphenol Content
UV	: Ultraviolet
WG	: Wheat Germ

CHAPTER 1

INTRODUCTION

1.1 WHEAT

Wheat is grown on more land area worldwide than any other cereal crop and is a close third to rice and corn in total world production [WORC, 2002]; it is one of the most important foods sold in the market. It was one of the first grains domesticated by man. The cultivation of wheat is thought to have had its origin in the Fertile Crescent of Middle East, carbonized remains of wheat grains and imprints of grains in baked clay have been found in the Neolithic site of Jarno in northern Iraq having an estimated radiocarbon date of 6700 B.C. [Inglett, 1974]. Also studies by Mangelsdorf suggest that wheat had its origin in the Caucasus-Turkey-Iraq area [Huges et al., 1957].

1.2 CLASSIFICATION OF WHEAT

Wheat belongs to the grass family Gramineae (Poaceae) and the genius Triticum [Wilson, 1955]. The two important groups from that genus are:

Triticum vulgare (aestivum): It is also called the common wheat. Aestivum is the most widely cultivated form of wheat and it is used for bread making.

Triticum durum: Sometimes called macaroni wheat but more correctly referred to as durum wheat [Wilson, 1955].

The market classification of the wheat is based upon the uses made of different types and does not necessarily bear any relationship to their botanical groupings [Wilson, 1955]. This classification varies from country to country mainly color; hardness and session are important items for classification. The American classification, which is commonly accepted worldwide, is given in Table 1.

Table 1 American classification of wheat

Type of Wheat	Protein Content	Purpose of Usage
Hard Red Spring	High	Bread, hard baked good
Hard Red Winter	Very high	Bread, hard baked good, supplement to other flours to increase the protein content. The best wheat for bread making.
Soft Red Winter	Medium	Bread and baking, pastry
Durum	Highest	Used to make pasta
Red Durum	Highest	Not very popular have no place in pasta manufacture
White	Medium	Bread and brewing
Mixed	Low	Bread, baking

However the classification of wheat in Turkey slightly differs from the American version. In Turkey there are nine classes of wheat, which are based

on the region of planting, hardness, color and shape of kernels. These classes are:

Milling Wheat:

- Anatolian Hard White
- Anatolian Red White
- Semi Hard Red
- Semi Hard White
- Others (White - Red)
- Feed Wheat

Durum Wheat:

- Anatolian Durum
- Other Durum
- Low Quality Durum

Hard wheats are higher in protein and gluten content so they are usually used for making breads. Soft wheats are used in the patisseries. All-purpose flour is made from soft and hard wheats.

Pasta or macaroni is made from durum wheat. So it is sometimes called as the "Pasta Wheat".

1.3 WORLD WHEAT PRODUCTION

Wheat is an important cereal crop. The worldwide wheat production for the year 2003 is almost 600 million tons. Wheat is well adapted to harsh environments and is mostly grown on everywhere any time. Wheat is harvested somewhere in the world in nearly every month of the year [Pomeranz, 1987]. Worldwide wheat production for the year 2003 is graphically illustrated in Figure 1[1].

Top five wheat producers in the world are:

- China
- India
- United States
- France
- Russia

[1] Food And Agriculture Organization Of The United Nations (FAO) (www.fao.org), http://www.fao.org/docrep/005/y9141e/y9141e06.htm at 2004-09-18

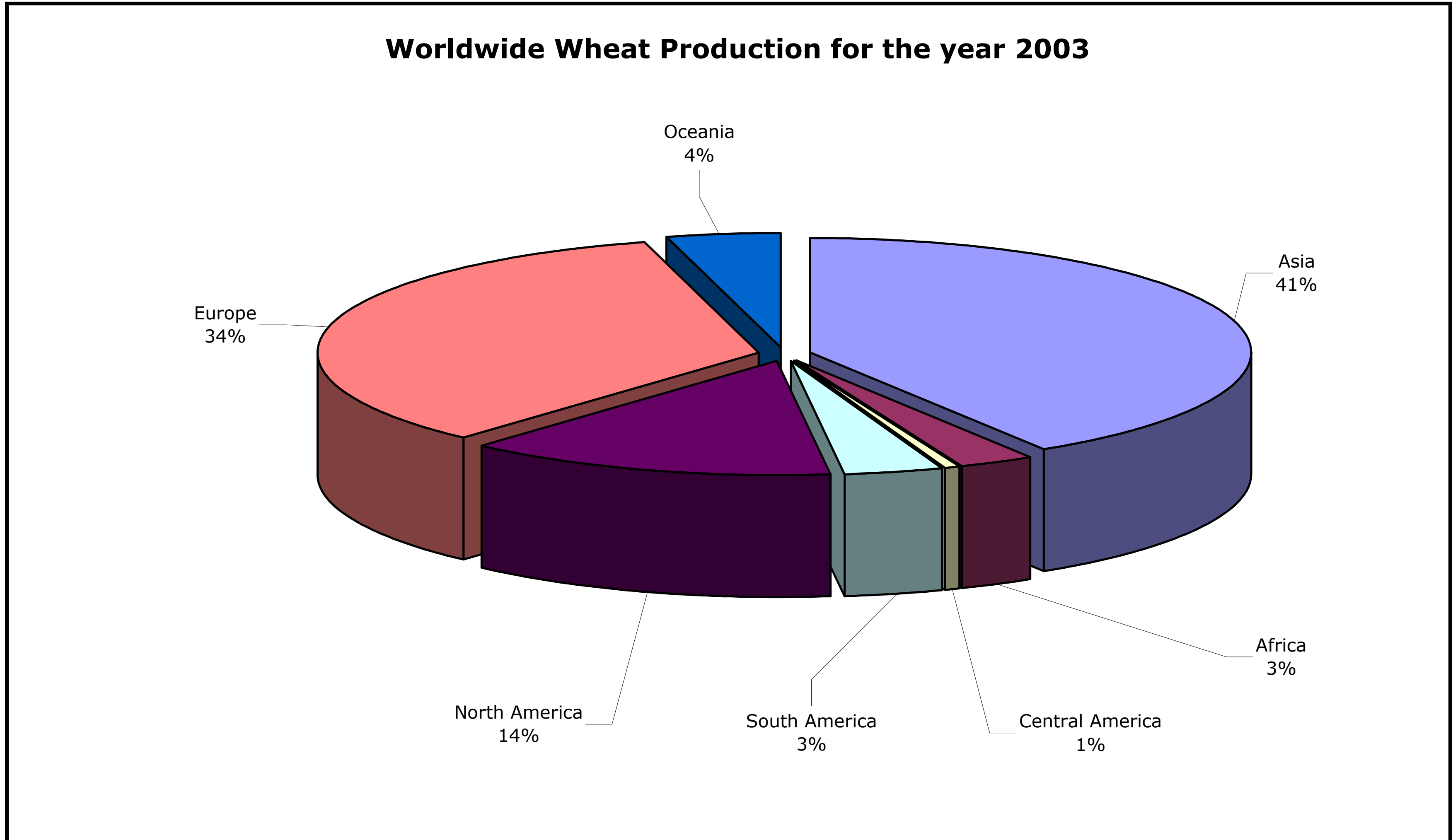

Figure 1 Wheat production in the world for the year 2003

1.4 WHEAT PRODUCTION IN TURKEY

Wheat is the most important cereal crop domesticated in Turkey. It is estimated that for the year 2004; 5.000.000 tons of durum and 16.000.000 tons of milling wheat will be produced in Turkey [DİE, 2004]. Turkey covers nearly 3% of the world wheat production. It is cultivated nearly everywhere except the Black Sea Region; Figure 2 shows wheat and durum wheat production in Turkey with other important cereal crops. Some basic statistics about Turkey's wheat production for the last two decades are given on Table 2.

Table 2 Statistics about Turkey's wheat production[2]

Wheat	**1980**	**1985**	**1990**	**1995**	**2000**
Production (tons)	16 554 000	17 032 000	20 022 000	18 015 000	18 000 000
Harvested area (ha)	8 956 000	9 274 500	9 432 309	9 400 000	8 650 000
Yield (kg/ha)	1848	1836	2122	1916	2080
Import (tons)	-	781 923	2 180 731	1 253 331	963 000
Export (tons)	338 049	268 923	24 975	232 847	1 782 048
Consumption (kg/person/yr)	2010	2076	2018	1970	1874

[2] Food And Agriculture Organization Of The United Nations (FAO) (www.fao.org), http://www.fao.org/ag/AGP/AGPC/doc/field/Wheat/asia/turkey.htm at 2004-09-18

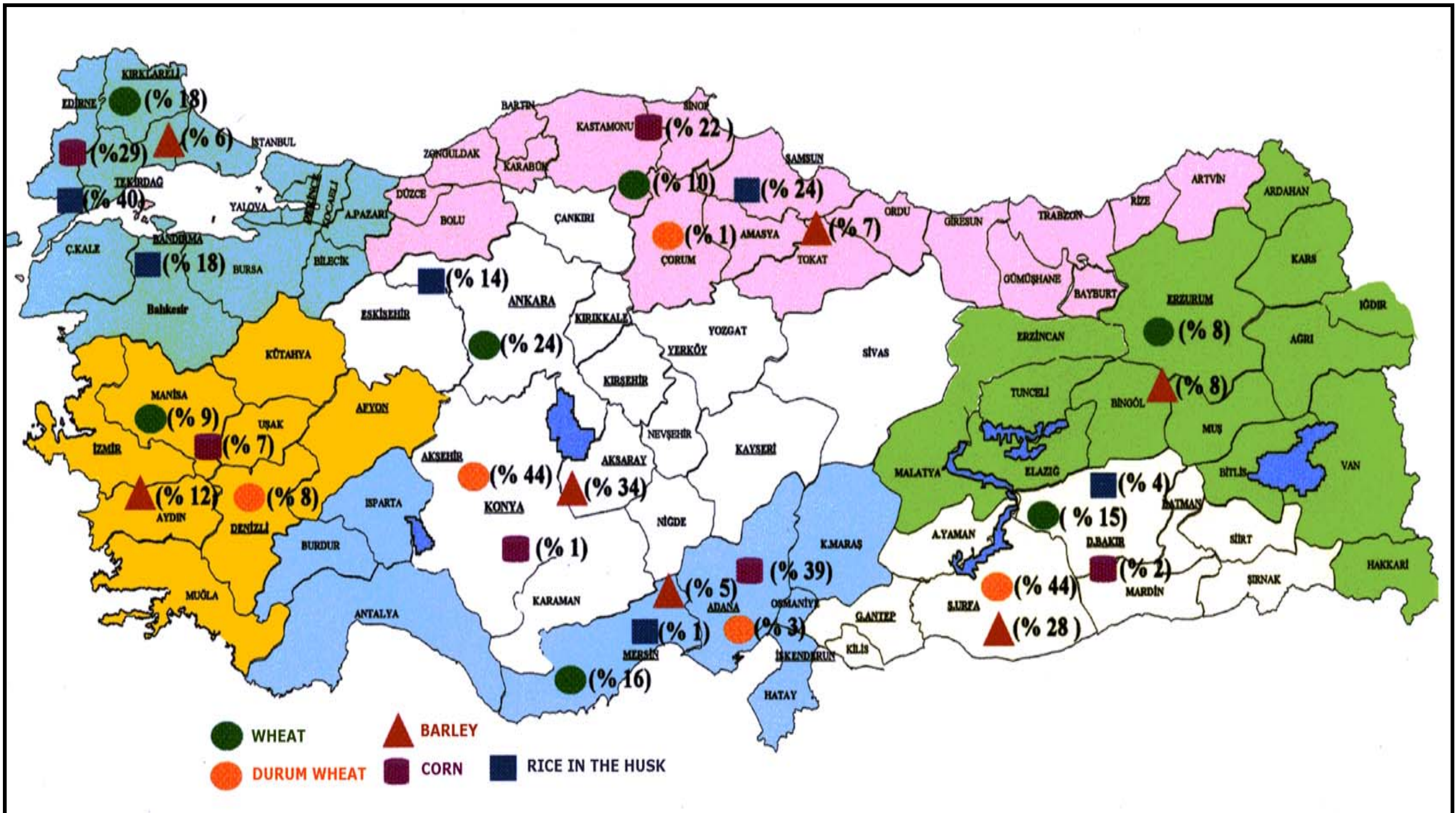

Figure 2 Crop production in Turkey [DİE, 2004]

1.5 WHEAT KERNEL (GRAIN)

The wheat kernel or grain is the seed from which the wheat plant grows. It is botanically known as caryopsis and is about 4-8 mm long, depending on the variety and condition of growth [Cornell et al., 1996]. Wheat kernel contains essential nutrients for human diet. Each kernel contains three parts; the endosperm, bran and the germ. The distribution of weight, protein and starch in these three parts of the kernel are given in Table 3. During the milling process to produce flour these parts are separated from each other.

Table 3 Distribution of weight, protein and starch in the wheat kernel[3]

Part	Fraction of kernel	% of kernel weight	% of total starch	% of total protein
Bran	Pericarp, testa	8	0	4.5
	Aleurone	7	0	15.5
Endosperm	Endosperm	82.5	100	72.0
Germ	Scutellum	1.5	0	4.5
	Embryo	1	0	3.5

[3] The Regional Institute Ltd. Online Community Publishing (www.regional.org.au), http://www.regional.org.au/au/roc/1988/roc198823.htm at 2004-09-14

1.6 ENDOSPERM

The endosperm contains the food material that the plant needs until it develops a complete root system. It comprises nearly eighty three percent of the weight in the kernel and contains nearly all the carbohydrates.

The endosperm contains the highest percentage of the protein in the kernel with 72 percent. The endosperm is also rich in riboflavin, niacin, and thiamine.

White flour is produced using only the endosperm of wheat, which is obtained by removing the seed coats and the germ in the milling process.

1.7 BRAN

Bran makes up 14 – 15 percent of the kernel weight. It contains pericarp, testa and the aleurone cells. Pericarp is a tough skin, it protects the inner seed from the environment and the inner seed coats control the water intake.

Dietary fiber, which is an indigestible cellulose material, is found in the bran. Fibers are necessary for getting rid of toxins and wastes from the body. And dietary fiber is one of the best fibers.

1.8 WHEAT GERM

The germ is where the life of a new wheat plant begins; thus it contains all the essential elements that are needed to begin this process. The germ comprises about two to three percent of the kernel weight.

Wheat germ is highly nutritious. It is a good source of protein, vitamin B, E and many minerals. The bulk and mineral compositions of wheat germ are given on Table 4 & 5 respectively.

Table 4 Composition of the crude wheat germ [NAL, 2004]

Nutrient	Value per 100 grams of edible portion
Proximate	
Water	11.12 g
Energy	360 kcal
Protein	23.15 g
Total lipid (fat)	9.72 g
Ash	4.21 g
Carbohydrate, by difference	51.80 g
Fiber, total dietary	13.2 g

Table 5 Mineral composition of the crude wheat germ [NAL, 2004]

Nutrient	**mg per 100 grams of edible portion**
Minerals	
Calcium, Ca	39
Iron, Fe	6.26
Magnesium, Mg	239
Phosphorus, P	842
Potassium, K	892
Sodium, Na	12
Zinc, Zn	12.29
Copper, Cu	0.796
Manganese, Mn	13.301

Despite all of its nutritive properties wheat germ is a by-product of the wheat milling industry. Since it is very rich in unsaturated fatty acid content it can easily go rancid. And rancidity keeps the quality of the flour low. So this highly nutritious food is separated from the kernel in the milling process.

1.9 WHEAT TO FLOUR (SEPARATION OF THE KERNEL)

Since prehistoric times wheat has been milled to separate the outer bran and germ from the principal part of the grain, the endosperm [Inglett, 1974]. Flour milling has advanced from primitive and laborious household task to vast and sophisticated, to a large extent automated industry [Pomeranz, 1987].

Since white flour is produced from the endosperm the aim of milling industry is to separate it from the bran and the germ successfully. Wheat flour production involves wheat selection and blending, cleaning, conditioning, breaking, bolting or sieving, purification, reduction, and treatment (bleaching, enrichment, supplementation) [Pomeranz, 1987]. However, bleaching is not done in Turkey.

Bran is removed from the endosperm because it reduces gluten development. And the oil in the germ is highly rich in unsaturated fatty acid content, which can easily go into rancid, thus makes the quality of the flour low so it is also removed during the milling processes.

White flour is made only from the endosperm of the wheat kernel. However, when the bran and germ are removed from the wheat kernel, vitamins, minerals are decreased and dietary fiber is also removed from the composition of the flour. The chemical composition of the whole wheat (contains bran, germ and endosperm) and white wheat flour (contains only the endosperm) are given on Table 6 for comparison.

Table 6 Flour compositions[4]

Food	Energy (kcal)	Protein (g)	Fat (g)	Ca (mg)	Fe (mg)	Thiamine (mg)	Riboflavin (mg)	Niacin (mg)
Wheat, whole	323	12.6	1.8	36	4.0	0.30	0.07	5.0
Wheat flour, white	341	9.4	1.3	15	1.5	0.10	0.03	0.7

1.10 WHEAT GERM OIL

Wheat germ approximately makes up two to three percent of the weight of the whole kernel. Generally it contains 9 – 12 percent oil. Physical properties and chemical composition of wheat germ oil are given in Table 7 & 8 respectively.

Table 7 Physical properties of wheat germ oil [NAL, 2004]

Appearance	Oily amber liquid / vegetal odor / Yellow
Solubility in Water	Insoluble
Boiling Point	> 300º C
Specific Gravity at 25º C	< 1.0 or (0.93 - 0.94)
Refractive index	1.469-1.478

[4] Food And Agriculture Organization Of The United Nations (FAO) (www.fao.org), http://www.fao.org/docrep/W0073E/w0073e06.htm at 2004-09-18

Table 8 Chemical composition of wheat germ oil [NAL, 2004]

Nutrient	**Value / 100g edible portion**
Proximate	
Energy	3699 kj
Total lipid (fat)	100.00 g
Vitamins	
Vitamin E (alpha-tocopherol)	149.40 mg
Vitamin K (phylloquinone)	24.7 mcg
Lipids	
Fatty acids, total saturated	18.800 g
14:0	0.100 g
16:0	16.600 g
18:0	0.500 g
Fatty acids, total monounsaturated	15.100 g
16:1 undifferentiated	0.500 g
18:1 undifferentiated (oleic acid)	14.600 g
Fatty acids, total polyunsaturated	61.700 g
18:2 undifferentiated (linoleic acid)	54.800 g
18:3 undifferentiated (alpha linolenic acid)	6.900 g
Phytosterols	553 mcg

As it is seen from Table 8 above, wheat germ oil is very rich in unsaturated fatty acid content, mainly linoleic acid and alpha linolenic acid content. Structural formula of linoleic and linolenic acid are given in Figures 3 and 4.

Typically 55% wheat germ oil is linoleic acid and 7% is alpha linolenic acid.

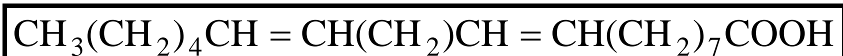
$$CH_3(CH_2)_4CH = CH(CH_2)CH = CH(CH_2)_7COOH$$

Figure 3 Structural formula of linoleic acid

These two polyunsaturated fatty acids are very important for human diet because human metabolism cannot create them from other fatty acids. In general these type fatty acids are called Essential Fatty Acids (EFA).

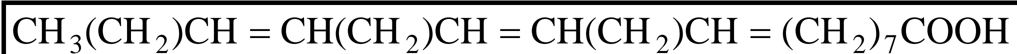
$$CH_3(CH_2)CH = CH(CH_2)CH = CH(CH_2)CH = (CH_2)_7COOH$$

Figure 4 Structural formula of linolenic acid

Wheat germ oil is the richest biological source of vitamin E [USDA Handbook #8], which is a natural antioxidant. Detailed information about vitamin E and antioxidants are explained on the next section.

Wheat germ oil is very valuable because of its chemical composition. It can be used in the following industries,

- Cosmetics, in creams, soaps and antiaging products,
- Food supplement, as it contains vitamin E, linoleic & linolenic acids.

Since wheat germ is not used in the production of white flour it is a by-product of wheat milling industry, which is mainly used as animal feed.

However this highly nutritious food material can be used to produce wheat germ oil, which is a marketable product, and can be sold in the market.

For the year 2004 it is estimated that 21.000.000 tons of wheat will be produced in Turkey. 16.000.000 tons of this wheat will be milling wheat and the remaining 5.000.000 tons will be durum.

From 21.000.000 tons of wheat, a rough estimate of theoretical wheat germ oil production can be done.

Assumptions:

- 2 % of this wheat is germ.
- An average of 10-percentage oil is found in these germs.
- And all of this oil can be extracted.

By simple mathematics,

$$\boxed{21.000.000 * \frac{2}{100} * \frac{10}{100} = 42.000\, tons\ of\ wheat\ germ\ oil}$$

Only for the year 2004 it is estimate that 42.000 tons of wheat germ oil is thrown away. And approximately 40.000 - 45.000 tons of wheat germ oil is thrown away every year.

However this oil must be extracted from the wheat germ. Extraction can be defined as the process of recovering a certain analyte found in a certain sample and purifying that analyte.

Extraction of wheat germ oil is considered under the hood of the title extraction of carrier oils. Detailed explanation of extraction techniques are explained in Chapter 2.

1.11 ANTIOXIDANTS, VITAMIN E and POLYPHENOLS

According to a very general definition antioxidants are substances capable of delaying, retarding or preventing oxidation processes [Schuler, 1990].

"In 1922 the biochemists Herbert Evans and Katharine Bishop found that a diet of rancid fat almost completely halted reproduction in rats. Only when wheat – germ oil was added to the diet did reproduction rates return to normal. In 1925 Evans named the substance responsible for this effect vitamin E" [Roche, 2004].

As it is mentioned in the previous section, wheat germ oil is the richest biological source of vitamin E, which is a natural antioxidant. Vitamin E contents of some food sources are given in Table 9 in descending order.

Table 9 Selected food sources of vitamin E[5]

FOOD	Milligrams (mg) Alpha-tocopherol per serving	Percent DV*
Wheat germ oil, 1 tablespoon	20.3	100
Sunflower oil, over 60% linoleic, 1 tablespoon	5.6	30
Safflower oil, over 70% oleic, 1 tablespoon	4.6	25
Peanut butter, smooth style, vitamin and mineral fortified, 2 tablespoon	4.2	20
Corn oil (salad or vegetable oil), 1 tablespoon	1.9	10
Soybean oil, 1 tablespoon	1.3	6
Kiwi, 1 medium fruit without skin	1.1	6
Spinach row, 1 cup	0.6	4

* DV = Daily Value. Daily values are reference numbers developed by the Food and Drug Administration (FDA) to help consumers determine if a food contains a lot or a little of a specific nutrient.

The benefits of vitamin E for the human body are:

- It helps the immune system functions,
- It protects fatty acids against oxidative damage,
- It protects cell membranes,
- And a key element to antiaging.

[5] U.S. Department of National and Health and Human Services, National Institutes of Health (www.nih.gov), http://dietary-supplements.info.nih.gov/ at 2004-10-10

Antioxidants such as vitamin E act to protect cells against the effects of free radicals. Free radicals are highly reactive oxygen compounds that are formed in the body mostly as by – products of respiration, but also as a result of ingestion of environmental and medicinal substances [Roche, 2004]. Free radicals can accelerate aging and can cause the development of various diseases.

The problem of oxidation is one of the most important problems in the food industry. Especially, in food preservation when a food product is oxidized an unpleasant taste and order develops, which both manufactures and consumers don’t want.

Ironically, wheat germ is separated from the wheat because of keeping the quality low, by spoiling easily. However wheat germ oil contains vitamin E, which is a natural antioxidant that can delay the spoilage of other food products or more importantly can be used as a food supplement since antioxidants protect the cells from effects of free radicals. So from a by – product of wheat industry an important food preservative and dietary supplement can be obtained.

One of the most important natural antioxidants are tocopherols. Tocopherols are substances having vitamin E activity. Physical properties of tocopherols are given in Table 10.

Table 10 Physical properties of tocopherols

Appearance	Oily substance
Color	Yellow
Solubility in Water	Insoluble
Solubility	Miscible at any ratio with vegetable oils, ethanol, acetone
Stability	Heat and acid stable however deteriorate with exposure to alkali, light or oxygen

There are eight different tocopherols found in the nature four of which occur naturally in foods, (alpha, beta, gamma and delta). For the vitamin E activity α-tocopherol is the most important form because it is the most active form of vitamin E in humans.

Commonly the amount of vitamin E found in a food product is expressed in terms of the α-tocopherol content. According to Table 8, 100 g of wheat germ oil contains approximately 150 mg of Vitamin E (alpha-tocopherol).

Alpha-tocopherol is mainly found in vegetable products, the highest amount is found in the wheat germ oil. However the content may depend very much on the variety and the growing conditions of the food product.

Although, there is no scientific evidence it is believed that antioxidants are answers to aging. In order to summarize the benefits of vitamin E, it is:

- An important antioxidant,

- Vital for the protection of nerve and muscle cell functions,
- Is important fertility.
- Deficiency can cause various diseases.

So vitamin E must be consumed adequately in the daily diet. As it is seen from Table 9, one tablespoon of wheat germ oil comprises 100% of Daily Value of a grown man. Thus its extraction is very important.

Another group of antioxidants are polyphenols, which are a group of vegetable chemical substances, characterized by the presence of more than one phenolic group or a group name to cover many different forms of phenolic compounds. "Alcohols have the general formula R – OH, and are structurally similar with water but with one of the hydrogens replaced by an alkyl group. Their functional group is the hydroxyl group, - OH. Phenols have the same functional group, but it is attached to an aromatic ring" [Hart et al., 1999].

"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 et al., 2000].

The main classes of the polyphenols, which man consume, are:

- Phenolic acids,
- Flavonoids, the most abundant polyphenols consumed in the human diet,
- And lignans.

Polyphenols are very important in the case of their antioxidant capacity. In literature the total antioxidant capacity is mainly expressed in terms of the total phenolic content generally in terms of gallic acid equivalent.

In the work done by Zheng and Wheng, total phenolic content of some selected herbs (i.e. Mexican oregano, Greek mountain oregano and hard sweet marjoram) and in the work done by Chu et al. 2002, total phenolic content of known vegetables (i.e. Broccoli, Spinach, Onion, Red Pepper, Carrot, Cabbage and Potato) were tabulated in terms of mg GA / g fresh weight. In the work done by Yu at al., 2003 wheat grains were grounded and extracted for 3 h with absolute ethanol under nitrogen, using a Soxhlet extractor. And the total polyphenol content was determined using the same method that was carried out in this thesis. In the work done by Zhou and Yu, 2003, different types of bran were ground to 80 mesh and extracted for 15 h with 20 ml with absolute ethanol, under nitrogen at ambient temperature. The ethanol extracts from Akron and Trego wheat bran's contain approximately 0.65 and 0.50 mg gallic acid / g bran respectively.

CHAPTER 2

LITERATURE SURVEY

2.1 EXTRACTION TECHNIQUES

The human body does not produce essential fatty acids such as linoleic and linolenic acids therefore it is important to get these nutrients through diet. Carrier oils or seed oils are found in the seed of the plants, which carry the essential oils. They don't vaporize easily and commonly solvent extracted. There are two types of solvent extraction:

1. Solvent extraction
    a. Solvent extraction (percolation)
    b. Maceration with solvent
2. Modern solvent extraction techniques
    a. Microwave Assisted Extraction
    b. Pressurized Solvent Extraction
    c. Supercritical Fluid Extraction
    d. Ultrasound Assisted Extraction

2.2 SOLVENT EXTRACTION

Classical extraction technologies are based on the use of an appropriate solvent to remove lipophilic compounds from the interior of plant tissues [Li et al., 2004].

The key element for the solvent extraction is obviously the solvent. For current studies the extraction solvents were chosen taking into consideration the following factors [Albu et al., 2004]:

- Polarity,
- Boiling point - this should be low in order to facilitate removal of the solvent from the product,
- Cost,
- Suitability for reuse,
- The solvent should be available in substantial quantities
- Safety in use – the solvent should, if possible, be non flammable and should not present a toxicity hazard to technicians or consumers; its disposal should not endanger the environment,
- Reactivity – the solvent should not react chemically with the extract, nor should it readily decompose.

Hexane is the most common solvent used for the extraction of carrier oils it obeys most of the factors above but it is hazardous and flammable.

Due to the hazards of hexane, for health and safety concerns, alternative solvents such as isopropanol, ethanol and supercritical carbon dioxide became more popular.

Wheat germ oil extraction is the main objective of this thesis work, thus extraction methods applied on plant materials will be explored in great concern rather than other product specific solvent extraction techniques.

A solvent extraction system is mainly composed of an extraction, filtration & evaporation, and a drying unit.

For analytical purposes there are various methods used for extraction. These analytical methods are:

- Soxhlet
- Microwave Assisted Solvent Extraction
- Pressurized Solvent Extraction
- Subcritical Water Extraction or Superheated Water Extraction
- Supercritical Fluid Extraction
- Ultrasonic Extraction

Ultrasonic extraction is the main extraction method used for the extraction of wheat germ oil in this thesis work, so before explaining it, brief information about the other extraction techniques are given on the following section.

2.3 SOXHLET (ANALYTICAL)

Soxhlet extraction was developed by Franz Soxhlet in 1879. Since that time, soxhlet has been a standard technique during more than one century and, at present; it is the main reference to which the performance of other leaching methods is compared [Luque de Castro et al., 1998].

The working principle of a soxhlet can be summarized as [Luque de Castro et al., 1998]: "In conventional Soxhlet, the sample is placed in a thimble – holder, and during operation gradually filled with condensated fresh solvent from a distillation flask. When the fluid reaches the overflow level, a siphon aspirates the solute of the thimble-holder and unloads it back into the distillation flask, carrying the extracted analytes into the bulk liquid. This operation is repeated until complete extraction is achieved. This performance makes Soxhlet a hybrid contionus – discontinous technique."

Still soxhlet extraction is the reference method for analytical extraction processes, maximum extraction can be done with soxhlet, because of the siphon action. This action, makes the sample in the extraction thimble is continuously exposed to fresh, heated solvent which increasing the extraction rate significantly. The advantages of the soxhlet are:

- The sample is repeatedly brought into contact with the fresh portions of the solvent, due to the siphon action.
- Simple to use.
- No filtration is required after the leaching step.

The most significant drawbacks of soxhlet extraction, as compared to the other conventional techniques for solid sample preparation are [Luque de Castro et al., 1998]:

- The long time required for the extraction
- The large amount of solvent wasted, which is not only expensive to dispose off but which can itself cause additional environmental problems.

2.4 MICROWAVE ASSISTED SOLVENT EXTRACTION (ANALYTICAL)

Microwaves produce an electric field that rapidly oscillates back and forth in direction; the field exerts oscillating torques on the molecules, continually rotating them back and forth to align dipole moments with the filed direction [Halliday et al., 1997]. The oscillations produce collisions with surrounding molecules and heat is liberated in the medium.

Microwave-assisted extraction (MAE) is a process of using microwave energy to heat solvents in contact with a sample in order to partition analytes from the sample matrix into the solvent [Pare, 1991].

The major advantage of the microwave assisted solvent extraction is the speed of heating. The sample in contact with the solvent is heated in seconds thus the extraction process is completed in minutes.

"According to the method patented by Pare. The samples were suspended in hexane and the microwaves reached the inner glandular and vascular systems of the plant material. Owing to high moisture content of these structures they were heated almost specifically and this promoted disruption of cell membranes releasing the analytes into the solvent" [Kaufmann et al., 2002].

There are two types of instruments commercially available for microwave assisted solvent extraction [Kaufmann et al., 2002]:

- Closed vessel; under controlled pressure and temperature,
- Open vessel or Microwave-assisted soxhlet extraction; under atmospheric pressure.

2.5 PRESSURIZED SOLVENT EXTRACTION (ANALYTICAL)

Solvating properties of a solvent increases with temperature. However under constant pressure there is a limit, if the solvent reaches its boiling point it turns into a gas and its extraction efficiency decreases to zero. Boiling point is defined at a certain pressure so if the pressure exerted on the solvent increases its boiling point must also increase. So by increasing the pressure the solvent is a liquid at a higher temperature and its solvating properties is increased.

Pressurized Solvent Extraction (PSE) increases a solvent's temperature, but keeps it a liquid by increasing pressure. Thus its solvating properties increased significantly.

2.5.1 SUBCRITICAL WATER EXTRACTION (ANALYTICAL)

Subcritical water extraction is a technique based on the use of water as extractant, at temperatures between 100 – 374 ° C (critical point of water, 22.4 MPa and 374°C) and pressures high enough to maintain the liquid state [Ayala et al., 2001]. As the temperature of liquid water is raised under pressure between 100 and 374 º C, the polarity decreases markedly and it can be used as an extraction solvent for a wide range of analytes [Smith, 2002].

2.5.2 ACCELERATED SOLVENT EXTRACTION (ANALYTICAL)

Accelerated solvent extraction (ASE) is a trademark; it is the first Pressurized Solvent Extraction instrument commercialized by Dionex Corporation in 1994.

2.6 SUPERCRITICAL FLUID EXTRACTION (ANALYTICAL)

The point of highest temperature at which a liquid can exist is the critical temperature, T_C and the pressure of this highest temperature is called the critical pressure, P_C.

A fluid above its critical pressure and temperature is called a supercritical fluid. In the supercritical state the distinction between the liquid and the gas phase has disappeared and the fluid can no longer be liquefied by raising the pressure nor can gas be formed on increasing the temperature [Sihvonen et al., 1999].

Supercritical fluid extraction (SFE) is an extraction method based on the enhanced solvating properties of supercritical fluids.

The most widely used fluid in SFE is carbon dioxide, CO_2, because it is:

- Non toxic,
- Can be obtained easily,
- Inexpensive,
- And non-flammable.

The solvent removal stage of the supercritical carbon dioxide extraction is the key behind the success of this technology. There is no need for an extra cleaning stage like in other methods, for supercritical carbon dioxide extraction; all needed is to remove the pressure under which it is kept.

2.7 ULTRASONIC EXTRACTION (ANALYTICAL)

2.7.1 ULTRASOUND

Ultrasounds are sound waves having frequencies greater than 20 kHz (above the human hearing). Although macrosonic effects extend well into the megahertz range (MHz), most practical applications to date have been in lower ultrasonic spectrum, between 20 to 60 kHz [Shoh, 1988].

Far before the commercial use of ultrasound, it has been utilized by animals over thousands of years. Bats use ultrasonic pulses in order to find their ways in the dark, likewise dolphins and whales use ultrasonic waves in order to find their mates in the oceans for reproduction.

Sound waves need material to travel, thus they involve expansion and compression cycles while they travel in this medium. In an expansion cycle molecules are moved apart from each other however in a compression cycle molecules are forced to come together.

"In a liquid, the expansion cycle produces negative pressure. If the ultrasound is strong enough the expansion cycle can create bubbles or cavities in the liquid. This is so when the negative pressure exerted exceeds the local tensile stress of the liquid, which varies depending on the nature and purity. The process by which vapour bubbles form, grow and undergo implosive collapse is known as cavitation. The whole process takes place within about 400 μs" [Luque-Garcia et al., 2003].

Normally, cavitation is a nucleated process, i.e. it occurs at pre-existing weak points in the liquid, such as gas-filled crevices in suspended particulate matter or transient microbubbles remaining from previous cavitation events. Most liquids are contaminated with sufficiently large amounts of small particles for cavitation to be readily initiated at fairly low negative pressure [Suslick, 1989].

At a certain time the bubbles cannot continue to grow so they collapse. Rapid adiabatic compression of gases and vapours in these bubbles or cavities can produce extremely high temperatures and pressures. Suslick et al [Suslick, 1994] estimated the temperature of these hot spots to be about 5000 º C that is similar to the surface of the sun and the pressure is roughly 1000 atm, which is equal to the pressure at the Marian Trench the deepest point of the ocean [Luque-Garcia et al., 2003].

However this extreme amount of heat produced cannot change the environmental conditions because the sizes of the bubbles are very small and the heat is dissipated in the medium in a very short period of time, i.e. cooling following a collapse of a bubble is estimated as 10 billion º C/s [Luque-Garcia et al., 2003].

Ultrasound waves with a low frequency, in the range of kHz, thus with high intensities are called high intensity or power ultrasound. Likewise ultrasound waves with MHZ range are called high frequency ultrasounds with low intensities. Ultrasound is used in different operations in chemical engineering: waste-water treatment, drying, sonochemistry and solid-liquid extraction [Romandhe et al., 2002]

2.7.2 ULTRASOUND ASSISTED EXTRACTION

Ultrasound Assisted Extraction is based on the destructive power ultrasonic waves. The possible benefits of ultrasound in extraction are [Vinatoru et al., 1999]:

- Mass transfer intensification,
- Cell disruption,
- Improved penetration
- And capillary effects.

Ultrasonic extractions of various analytes from different samples using different types of solvents are carried out in the literature. However for the work carried out in this thesis the main concern is to investigate the effect of ultrasound on the extraction wheat germ oil.

In case of raw plant tissues, ultrasound has been suggested to disrupt plant cell walls thereby facilitating the release of extractable compounds and enhance mass transport of solvent from the continuous phase into plant cells [Vinatoru, 2001].

The experiments concerning ultrasonically assisted extraction were carried out in three ways [Vinatoru et al., 1997]:

- Indirect sonication using an ultrasonic bath,
- Direct sonication using an ultrasonic horn,
- Direct sonication using an ultrasonic bath.

Ultrasonic bath and ultrasonic probe (or horn) are the two most common devices used for ultrasonication. Although ultrasonic baths are more widely used, they have two main disadvantages that substantially decrease experimental repeatability and reproducibility [Luque-Garcia et al., 2003]:

(a) lack of uniformity in the distribution of ultrasound energy (only a small fraction of the total liquid volume in the immediate vicinity of the ultrasound source experiences cavitation); and,

(b) decline of power with time, so the energy supplied to the bath is wasted.

Ultrasonic probes have their advantage over ultrasonic baths in the way they focus their energy on a localized sample one, thereby providing more efficient cavitation in the liquid [Luque-Garcia et al., 2003].

However as the cost item appears ultrasonic baths are much more cheaper than probes (horns) and they can be used for multiple operations at a single time. Also some ultrasonic baths have temperature controllers, which can be used to increase the extraction efficiency.

Ultrasonic extraction needs a wise clean up stage. Since the sample and the solvent are in direct contact they should be separated carefully.

- First of all while removing the sample from the solvent the desired analyte should be kept inside the solvent phase.
- Then this analyte should be separated from the solvent in a second stage.

For the extraction of wheat germ oil the method of indirect sonication using and ultrasonic bath was used.

2.8 COMPARISION OF THE ANALYTICAL EXTRACTION METHODS

The methods explained in this chapter were the most common analytical extraction methods found in the literature. The comparison of these techniques are given on Table 11 on the basis of the approximate extraction time, capital investment, sample size, solvent usage, advantages and disadvantages. Table 11 is a modified version of the work done by Eskilsson et al., 2000.

As it was seen from Table 11, ultrasonic assisted extraction using and ultrasonic bath is fast, cheap, allows multiple extractions in a single operation, allows high amount of samples to be extracted and gives moderate extraction efficiencies. But, it is not efficient as some of the techniques, like SFE and MAE.

However it was selected as the main extraction technique used in this thesis work because of the advantages listed above and more importantly it was not temperature dependent. The temperature inside the ultrasonic bath slightly changes through out the extraction process, between 0 – 60 min the temperature of distilled water at 25 º C raised only up to 40 – 45 º C.

Table 11 Comparison of the extraction techniques [adopted from Eskilsson et al., 2000]

EXTRACTION TECHNIQUE						
	SOXLET	**MICROWAVE ASSISTED EXTRACTION**	**MICROWAVE ASISTED SOXHLET EXTRACTION**	**PRESSURIZED LIQUID EXTRACTION (PLE)**	**SUPERCRTITICAL FLUID EXTRACTON (SFE)**	**ULTRASONIC EXTRACTION**
Approximate Extraction time	6 – 12 hours	3 – 30 min	10 – 60 min	5 – 30 min	10 – 60 min	10 – 60 min
Sample Size	1 – 30 g	1 – 10 g	1 – 30 g	1 – 5 g	1 – 30 g	1 – 30 g
Solvent Usage	100 – 500 ml	10 – 40 ml	10 – 150 ml	10 – 100 ml	2 – 5 ml (solid trap) 5 – 20 ml (liquid trap)	30 – 200 ml
Investment	Low	Moderate	Moderate	High	High	Low
Advantages	High extraction efficiency No filtration step	Fast Moderate Efficiency Multiple Reactions Low Solvent Consumption	Fast Moderate Efficiency Solvent reuse No filtration step	Fast Automatic system No filtration step	Fast No clean up and filtration stages are required Non hazardous solvent	Moderately Fast Multiple Extractions
Dis-advantages	Long extraction period Large amount of solvents are used Clean up stage is required	Solvent must absorb microwave energy Clean up and filtration stages are required Extra time needed for vessel cool down	Solvent must absorb microwave energy Clean up stage is needed	Clean up stage is needed	Small amount of samples can be extracted	Reproducibly Clean up and filtration stages are required

2.9 REASONS TO CHOOSE ULTRASOUND ASSISTED EXTRACTION

The main reasons of carrying out a thesis work on this subject were given below:

1. From the chemical engineering point of view wheat germ is an ideal raw material because:

   a) Wheat germ is a by-product of milling industry, which makes it widely available and cheap.
   b) It is very rich in essential fatty acid and polyphenol content.

2. For research:

   a) There was limited work found in the literature on the extraction of wheat germ oil. The most recent work is done by Dunford et al. 2003, which is about pressurized solvent extraction of wheat germ oil.
   b) No work has been found about ultrasonic extraction of wheat germ oil in the literature.

3. The best extraction method to study is the ultrasonic extraction because:

   a) It is temperature independent, as it is explained above,
   b) A fast extraction method,
   c) And the instrument of extraction, which is an ultrasonic bath, is cheap and can be used very easily.

The extraction of wheat germ oil the method of indirect sonication using an ultrasonic bath was used.

CHAPTER 3

MATERIALS & METHODS

3.1 MATERIALS

Wheat germs used in this study were obtained from Ankara Un Sanayi Anonim Şirketi. The germs were taken in three parties and classified accordingly, AUSAS June 2004, AUSAS July 2004, and AUSAS August 2004. The first two parties are directly used in tests however the last party was divided into two and half of it was roasted in order to see the effect of heat treatment. Wheat germs are shown in Figure 5. For the extraction purpose hexane and alternative solvents such as acetone, isopropanol and ethanol were used. All of the solvents used were at reagent grade.

Figure 5 Wheat germ

3.2 MOISTURE CONTENT

Moisture content is one of the most important characteristics of a food product. It is simply:

$$Moisture\ Content, \% = \frac{amount\ of\ water\ (g)}{weight\ of\ the\ food\ (g)} * 100$$

Moisture contents of the raw and roasted wheat germs were determined by drying at 105 º C for 2 hours as explained in the Appendix A1.

3.3 ASH CONTENT

The inorganic residue that remains after the removal of water and organic matter by heating in the presence of oxygen is called the ash. Ash is a measure of the total amount of minerals found in a food. Dry, wet and low temperature plasma ashing are three different methods used to measure the ash content. Dry ashing is the most convenient way of measuring the ash content.

In dry ashing a high temperature muffle furnace capable of maintaining temperatures of between 500 and 900 º C is used. Water and other volatile materials are vaporized and organic substances are burned in the presence of the oxygen in air to carbon dioxide and oxides of nitrogen.

Ash contents of the raw and roasted wheat germs were determined by keeping the sample at 900 º C for 4 hours using the procedure given in the Appendix A2.

3.4 FAT CONTENT

The fat content is very important since it shows the total amount of lipids found inside the germs. It was determined by soxhlet extraction for 6 hours with hexane using the procedure given in the Appendix A3.

3.5 STABILIZATION OF THE WHEAT GERMS

The half of the raw wheat germs obtained in the third party, AUSAS August 2004, were dried using a laboratory type spouted bed drier, Sherwood Scientific Equipment, which was shown in Figure 6.

Figure 6 Laboratory type spouted bed drier - Sherwood Scientific equipment

Three sets of samples each weight 150 g was dried according to the previous work done by Gürün et al., 2000, as follows:

1. The raw wheat germs were placed inside the glass tube of the drier,
2. A filter bag was fitted over the top of the tube,
3. Airflow rate was set to 11 L/s,
4. And for 6 minutes at 200 º C the roasting of the germs were carried out.

3.6 STORAGE OF THE WHEAT GERMS

Since wheat germ has a poor shelf life and can go rancid easily it must be stored very carefully.

Both the roasted and raw wheat germs were stored in airproof bags in deep freezers at –18 º C. From the previous work done by Gürün et al., 2000, it was decided that storing wheat germs at –18 º C, can cause slight or no rancidity. And for each experiment the required amount was taken and the remaining germs were placed into the freezers again.

3.7 ULTRASOUND ASSISTED EXTRACTION

The extraction of lipids and polyphenols from the wheat germs were carried out ultrasonically. The device used for the ultrasonic extraction is an ultrasonic bath, Bransonic 2200, with the following specifications given in Table 12.

Table 12 Technical specifications of the ultrasonic bath

Device	Bransonic Ultrasonic Cleaner
Model	B – 2200 E1
HF – Output power nom.	60 W
Working Frequency	47 kHz ± 6 &
Power Supply	120 W, 220 V, 50 – 60 Hz

As it was stated previously this type of extraction is classified as indirect sonication using an ultrasonic cleaning bath. The ultrasonic bath is shown in Figure 7.

Figure 7 Ultrasonic bath, Bransonic 2200

The ultrasound-assisted extraction carried through out this thesis work can be classified as follows:

1. Lipid Extraction:
   a. Determination of seed to solvent ratio, i.e. 1:1, 1:5.
   b. Determination of the most efficient solvent out of the three alternative solvents; acetone, isopropanol and ethanol.
   c. Determination of the ultrasonication time on the extraction efficiency, in the range of 0,5,10...40 minutes.
   d. Optimizing the way of further treatment or separation of the liquid (oil, solvent) and the solid (wheat germs and other solids) due to the usage of polar solvents. Combinations of putting into the refrigerator for a specified time, decantation, filtration and centrifugation.
2. Polyphenol Extraction:

The details of the ultrasound assisted extraction were given in the Appendix A4.

3.8 PHASE SEPARATION

In the first part of the experiments the separation of the solid and the liquid phases were carried out by decantation using the procedure given in the Appendix A5. However decantation has some drawbacks:

- First, the storage time in the refrigerator is very long, almost a day, which causes a time delay between successive analysis.
- Secondly, the phase separation is done by via decanting small amounts since some of the solid particles could remain in the liquid phase.

In literature the most accurate way to separate the solid and liquid phases is centrifugation. So in order to enhance the phase separation and analyze its effects on the extract yield, the effects of centrifugation and the duration inside the refrigerator were investigated in the following combinations:

1. The extracts in the erlenmeyer flasks were stored at +4 º C in a refrigerator for 18-24 hours and then filtrated.
2. The extracts were not stored at the refrigerator; nearly everything in the erlenmeyer flasks was poured into the centrifuge cartridge and centrifugation at 2800 rpm for 20 minutes were carried out. Then the liquid phase was poured through a funnel using a filter paper into another beaker.
3. Nearly everything in the erlenmeyer flasks was directly poured into the centrifuge cartridge after the extraction and centrifugation at 2800 rpm for 20 minutes was conducted. Afterwards the centrifuge cartridge was stored at +4 º C in a refrigerator for 18-24 hours. Then the liquid phase was poured through a funnel using a filter paper into another beaker.
4. The extracts in the erlenmeyer flasks were stored at +4 º C in a refrigerator for 18-24 hours. The liquid phase was gently poured into the centrifuge cartridge and centrifugation at 2800 rpm for 20 minutes was conducted. Then the liquid phase was poured through a funnel using a filter paper into another beaker.

3.9 SOLVENT EVAPORATION

In the analysis of the lipids and the determination of the percent extraction after the further treatment step the solvents were evaporated at 110 º C using the procedure given in the Appendix A6.

Solvent evaporation was a key step in the solvent extraction. Thus alternative solvents (acetone, isopropanol and ethanol) were selected instead of hexane which is hazardous. And the solvents were evaporated under the hoods using hot plates and masks were used all the time.

After the solvents were evaporated the beakers were set into the desiccators to cool down. Then the extract yields were calculated accordingly.

3.10 TOTAL POLYPHENOL CONTENT

For the total polyphenol content analysis the extracts of the roasted and raw germs of the party, AUSAS August 2004, were used. The germs were sonicated for 0, 5, 10, 15, 20, 25, 30, 35 and 40 minutes. Experiments were carried out in triplicate and the solutions were kept in dark between analysis.

The separation of the solid and liquid phases were done out by decanting using the procedure 4 given in Section 3.8.

The antioxidant capacity was measured in terms of the total polyphenol content. The procedure was adopted from the works of **Yu et al., 2002, Yu et al. 2003 and Waterhouse**, which was given in the Appendix A7. The composition of the Folin-Ciocalteu's phenol reagent used for the analysis was given in the Appendix D.

The summary of the experimental factors that were tested in this thesis work were given in Table 13.

Table 13 Experimental factors tested in the ultrasound assisted extraction

	Wheat Germ Type	Solvent Type	Extraction Technique	Germ to Solvent Ratio	Sonication Time	Further Treatment	Experiments Undertaken
Lipid Extraction	AUSAS June2004 (Raw)	Ethanol Isopropanol Acetone	Ultrasonic Bath	1:1, 1:2, 1:5, 1:10, 1:20	5 minutes	18 – 24 hour duration at +4 º C, Filtering, Evaporation	2 sets with Ethanol 2 sets with Isopropanol 2 sets with Acetone
	AUSAS July 2004 (Raw)	Ethanol Isopropanol Acetone	Ultrasonic Bath Soxhlet	1:20	0, 5, 10, 15, 20, 25, 30, 40 minutes	18 – 24 hour duration at +4 º C, Filtering, Evaporation	2 sets with Ethanol 2 with Isopropanol 2 sets with Acetone
	AUSAS August 2004 (Raw) AUSAS August 2004 (Roasted)	Ethanol	Ultrasonic Bath	1:10	0, 5, 25 minutes	18 – 24 hour duration at +4 º C, Filtering, Evaporation No duration, centrifugation at 2800 rpm for 20 min., Filtering, Evaporation Centrifugation at 2800 rpm for 20 min., 18 – 24 hour duration at +4 º C, Filtering, Evaporation 18 – 24 hour duration at +4 º C, Centrifugation at 2800 rpm for 20 min., Filtering, Evaporation	2 sets with raw germs 2 sets with roasted germs
Polyphenol Extraction	AUSAS August 2004 (Raw) AUSAS August 2004 (Roasted)	Ethanol	Ultrasonic Bath	1:10	0, 5, 10, 15, 20, 25, 30, 40 minutes	18 – 24 hour duration at +4 º C, Centrifugation at 2800 rpm for 20 min., Filtering,	3 sets with raw germs 3 sets with roasted germs

3.11 DATA ANALYSIS

The experimental data was analyzed using the data analysis tool of Microsoft Excel Analysis ToolPak. The work done can be summarized as follows [Draper et al., 1966]:

1. First a linear regression or a straight line was fit to the data points using Least Squares Technique.
2. Then an F – Test was carried out to see the significance of the regression. The F – Test has two outcomes:
   a. If $F < F_{CRITICAL}$ (which is tabulated in the literature) then the linear regression is not significant. Thus the mean of the data points express the data sequence better then any other linear regression.
   b. If $F > F_{CRITICAL}$ then a LOF (Lack of Fit) test was done. The LOF test has also two outcomes.
      i. If the F ratio is smaller then the tabulated F (1, n-2) distribution then the LOF was insignificant and on the basis of this test there is no reason to doubt the adequacy of the model.
      ii. If the F ratio is greater then the tabulated F (1, n-2) distribution then the LOF was significant so another model should be tested may be a polynomial one.

The results from the software package were tabulated in the Appendix B.

CHAPTER 4

RESULTS & DISCUSSION

4.1 ANALYSIS OF THE WHEAT GERMS

The moisture, ash and fat content of the raw and roasted wheat germs were given in Table 14, 15 and 16 respectively.

Table 14 Moisture content of the wheat germs

Sample	Moisture %			Average Moisture %	
AUSAS June 2004	10.9	11.0	11.1	11.0	10.8
AUSAS July 2004	10.4	10.2	10.6	10.4	
AUSAS August 2004 (Raw)	11.2	10.7	11.4	11.1	
AUSAS August 2004 (Roasted)	10.6	9.8	11.2	10.5	10.5

Table 15 Ash content of the wheat germs

Sample	Ash %			Average Ash %
AUSAS August 2004 (Raw)	3.9	3.9	4.1	4.0
AUSAS August 2004 (Roasted)	3.8	4.0	3.6	3.8

Table 16 Fat content of the wheat germs

Sample	Fat %	Average Fat %
AUSAS June 2004	11.3	11.1
AUSAS July 2004	9.8	
AUSAS August 2004 (Raw)	12.1	
AUSAS August 2004 (Roasted)	12.2	12.2

4.2 GERM TO SOLVENT RATIO

The solid to liquid or the seed to solvent ratio was an important factor that should be determined at the beginning. Since the other experimental factors were checked while keeping this ratio constant.

In order to determine the best ratio, preliminary extractions were taken using 1 gram of sample as the basis and changing the amount of solvent in a 50 ml erlenmeyer flask. For the ratios 1:1, 1:2, 1:5, 1:10 and 1:20 and for three alternative solvents, acetone, isopropanol and ethanol the extractions

were carried out. The experiments were done in duplicate and the averages were taken.

The extraction procedure given in the Appendix A4 was conducted for the specified parameters then the extracts obtained are decanted using the procedure given in the Appendix A5. Finally the solvent was evaporated and the extract ratio was calculated using the procedure given in the Appendix A6.

For the ratios 1:1, 1:2 and 1:5 it was seen that the germs and the solvent inside the erlenmeyer flasks were not mixed homogenously. And the extract yields were insignificant. The average extract ratios for three alternative solvents for different seed to solvent ratios were given on Table 17. As it was seen from Table 17 for the ratios of 1:10 and smaller, well mixing was observed and higher yields were achieved. So for the rest of the experiments 1:10, 1:20 and 1:30 ratios were used for convenience.

Table 17 Average extract ratios for different wheat germ to solvent ratios

Germ to Solvent Ratio	**Ultrasonication time (min)**	**Extract Ratio % (Average)**		
		Acetone	**Isopropanol**	**Ethanol**
1:1	5	Insignificant	Insignificant	Insignificant
1:2	5	Insignificant	Insignificant	Insignificant
1:5	5	Insignificant	Insignificant	5.6
1:10	5	2.9	2.7	7.6
1:20	5	3.3	2.6	8.9

4.3 EXTRACTION

The extractions were carried out using the second party of the wheat germs AUSAS July 2004. The aim was:

- To see the extraction profile and
- To determine the most efficient solvent

The extraction procedure explained in the Appendix A4 was conducted for the specified parameters:

- Germ: AUSAS July 2004, nonroasted
- Solvent: Acetone, Isopropanol and ethanol (in order)
- Germ to Solvent Ratio: 1 gram to 20 ml acetone
- Sonication time: 0, 5, 10, 15, 20, 25, 30, 35 and 40 min.

The extracts obtained are decanted, the solvent was evaporated and the extract yield was calculated in the manner of the procedures given in the Appendix A5 & A6. Experiments were carried out in duplicate and the data were given in the Appendix B. The extract ratio was calculated as follows:

$$Extract\ Ratio, \% = \frac{amount\ of\ \ extract\,(g)}{weight\ of\ \ germ\,(g)} * 100$$

Hexane is the most common solvent used for the extraction of carrier oils but it is hazardous. Thus it was only used for the fat content determination in the soxhlet.

For the extraction purpose alternative solvents such as acetone, isopropanol and ethanol were used. The extract yields obtained with these solvents were relatively high however the extracts obtained were cloudy.

The alternative solvents were polar in nature so they act differently than hexane. While hexane dissolves only the fats, these solvents also dissolve wax, phospholipids and some other proteins. So the extraction could be defined as the lipid extraction since the major components were in the lipid phase.

And this naturally occurring phenomenon was very beneficial because it is known that wax and phospholipids can be added to creams and soaps. Thus extraction of these substances together with the oil was very important. So the yields were expresses as the "extract ratio" but not as the "fat content".

4.3.1 EXTRACTION WITH ACETONE

For acetone a linear regression was tried to be found between the extract ratio and ultrasonication time. However, from the F –Test it was seen that $F = 0.47$ which was smaller then the $F_{CRITICAL}$, which means that the linear regression was insignificant. Thus the mean represents the data points better than any other regression and it was concluded that the extract ratio was not a linear function of time. Also the mean of extract ratios which is equal to 3.4 was quite small. Therefore acetone was not a good solvent for the ultrasonic extraction process.

The experimental data was given in the Appendix B1 and the results were given in Figure 8. Meanwhile, the results of the statistical analysis were tabulated in the Appendix B1.1 and in Figures 19 and 20 respectively.

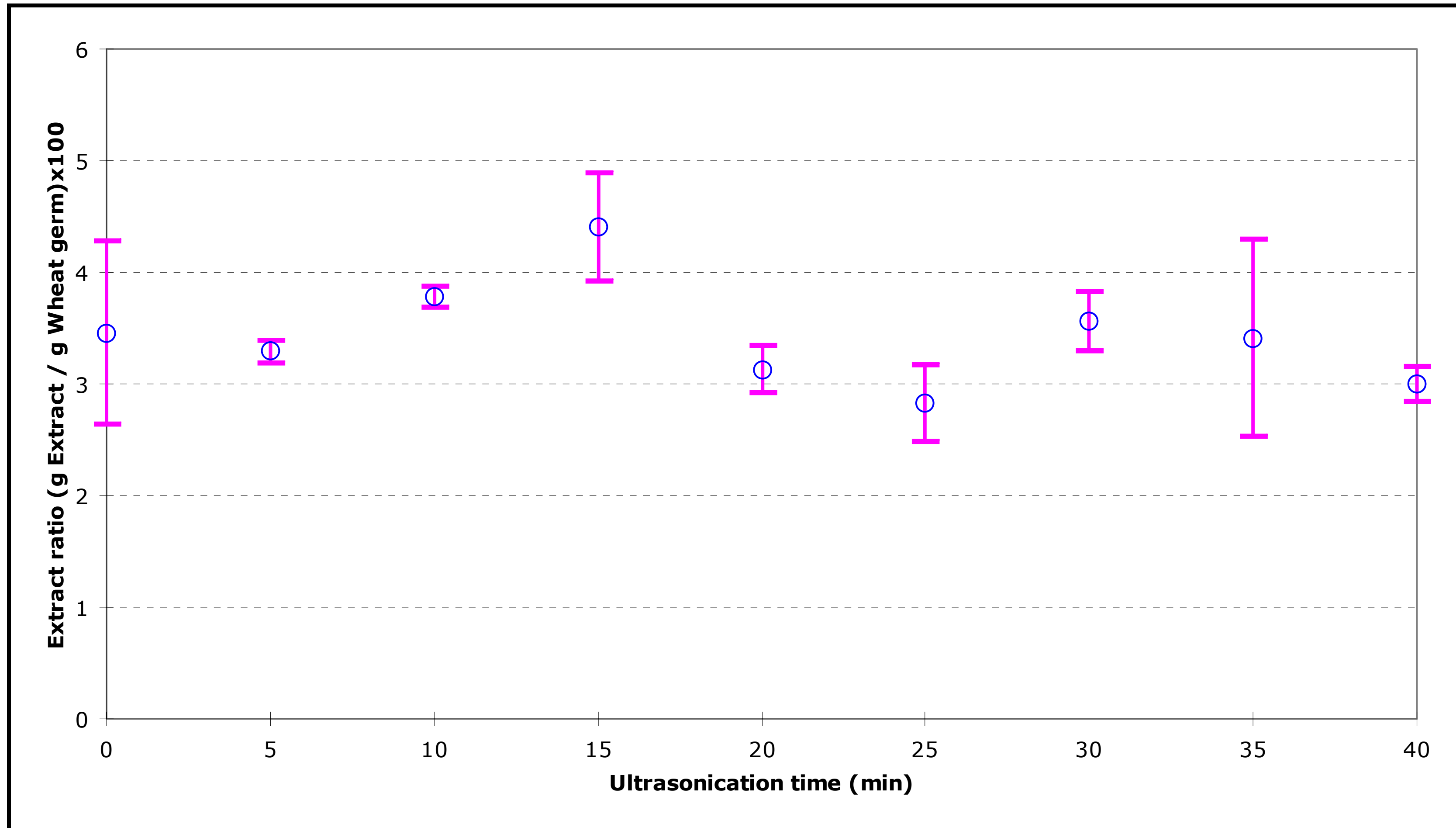

Figure 8 Extract Ratio vs. Ultrasonication time graph of acetone

4.3.2 EXTRACTION WITH ISOPROPANOL

For isopropanol a linear regression was tried to be found between the extract ratio and ultrasonication time. And from the F –Test it was seen that $F = 5.5 > F_{CRITICAL}$ and from the LOF test it was seen that $F_{LOF} < F_{CRITICAL}$ which means lack of fit was insignificant. Thus there was no reason to doubt the adequacy of the model. The linear regression had a slope = 0.07 with R^2 = 0.26. Thus the extract ratio was expressed as a function of time:

$$E = 0.07*t + 2.2 \text{ with } R^2 = 0.26 \text{ where,}$$

E = Extract Yield %

t = time, min.

However, the highest extract ratio obtained through out the experiments was 6.6 % and the average of the extract ratios was 3.4 which were small. So isopropanol was not a good solvent for the ultrasonic extraction process.

The experimental data and statistical analysis were given in the Appendix B2 and the results were given in Figure 9. Meanwhile, the results of the statistical analysis were tabulated in the Appendix B2.1 and in Figures 21 and 22 respectively.

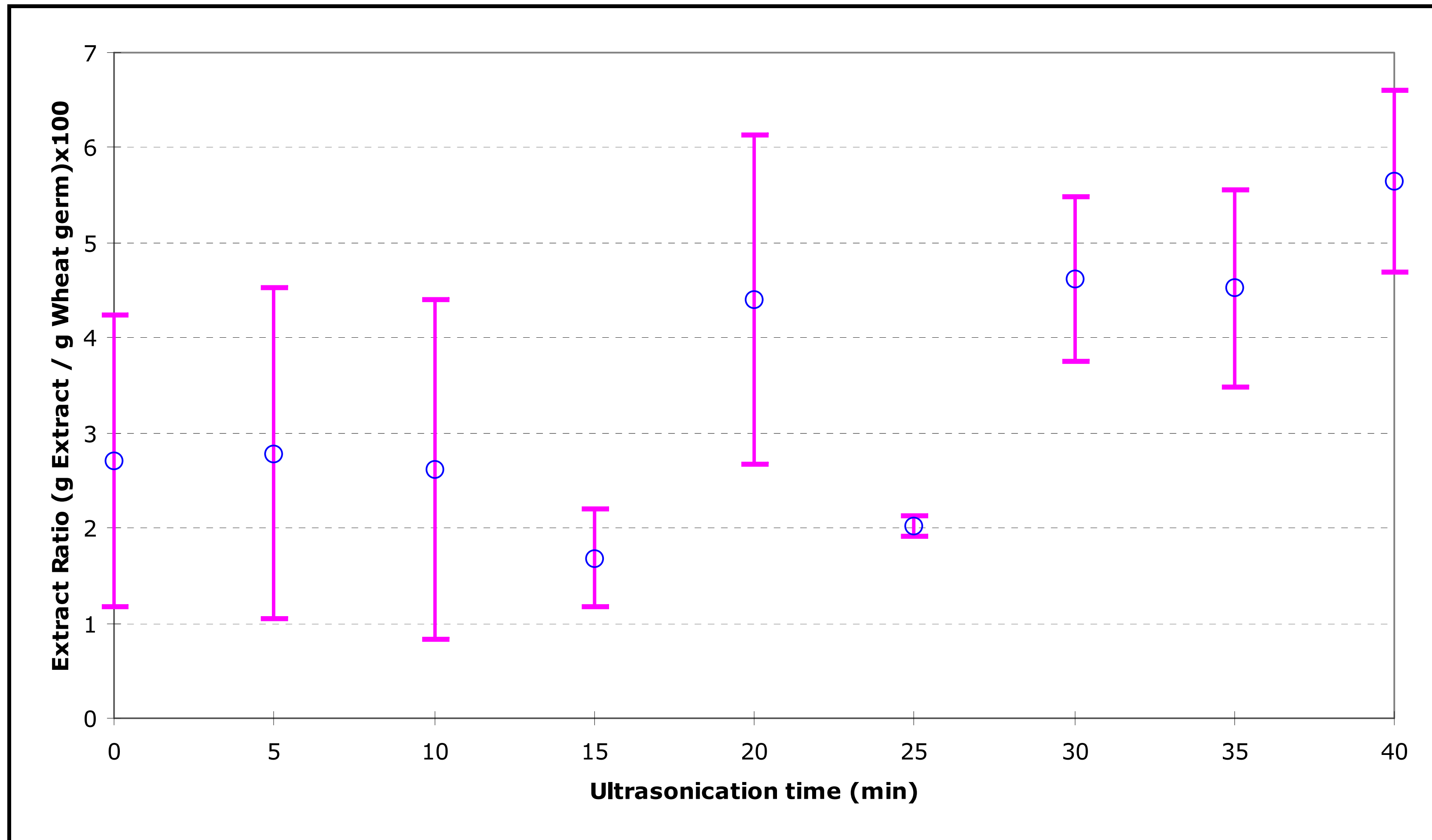

Figure 9 Extract Ratio vs. Ultrasonication time graph of isopropanol

4.3.3 EXTRACTION WITH ETHANOL

The experimental data and statistical analysis were given in the Appendix B3 and the results were given in Figure 10. Meanwhile, the results of the statistical analysis were tabulated in the Appendix B2.1 and in Figures 23 and 24 respectively. And the comparison of the extract yields of three alternative solvents was given in Figure 11 and 25.

After the analysis with acetone and isopropanol it was decided to use ethanol. For ethanol a linear regression was tried to be found between the extract ratio and ultrasonication time. However from the F –Test it was seen that F = 0.44 which was smaller then the $F_{CRITICAL}$ which means that the linear regression was insignificant. Thus the mean represents the data points better than any other regression and it is concluded the extract yield was not a linear function of time. The mean of the data points were 9.51.

Although no significant linear regression was found between the extract yield and time for ethanol from Table 18 it was seen that the average yield was 280 % greater then acetone and isopropanol. Also it was known that for the total polyphenol analysis the most popular solvent used in the literature was ethanol. So it was decided to use ethanol for the remaining part of the experiments, mainly on the total polyphenol content analysis of the wheat germs.

For the extraction experiments all the extracts obtained after the sonication process were cloudy due their polar nature and the storage of these extracts in the refrigerator for 24 hours settled some of the solid particles. However the solids were not completely removed from the liquid phase, thus

some remained suspended in the liquid phase. Thus for the extraction with ethanol an average extract yield of 9.5 was achieved due to its polar nature and phase separation technique. However this extracts were obtained at relatively low temperatures and short period of ultrasonication, for 40 min of ultrasonication the temperature in the bath only increased to 40 º C. So ultrasonic extraction was temperature sensitive and gave relatively high extract ratios. Thus the aim of this work was not to separate the oil from the wheat germ in a perfect way but to extract phospholipids, polyphenols and waxes together with the oil in order to obtain a valuable product from a by product like wheat germ. Consequently, these goals were achieved with the usage of ethanol.

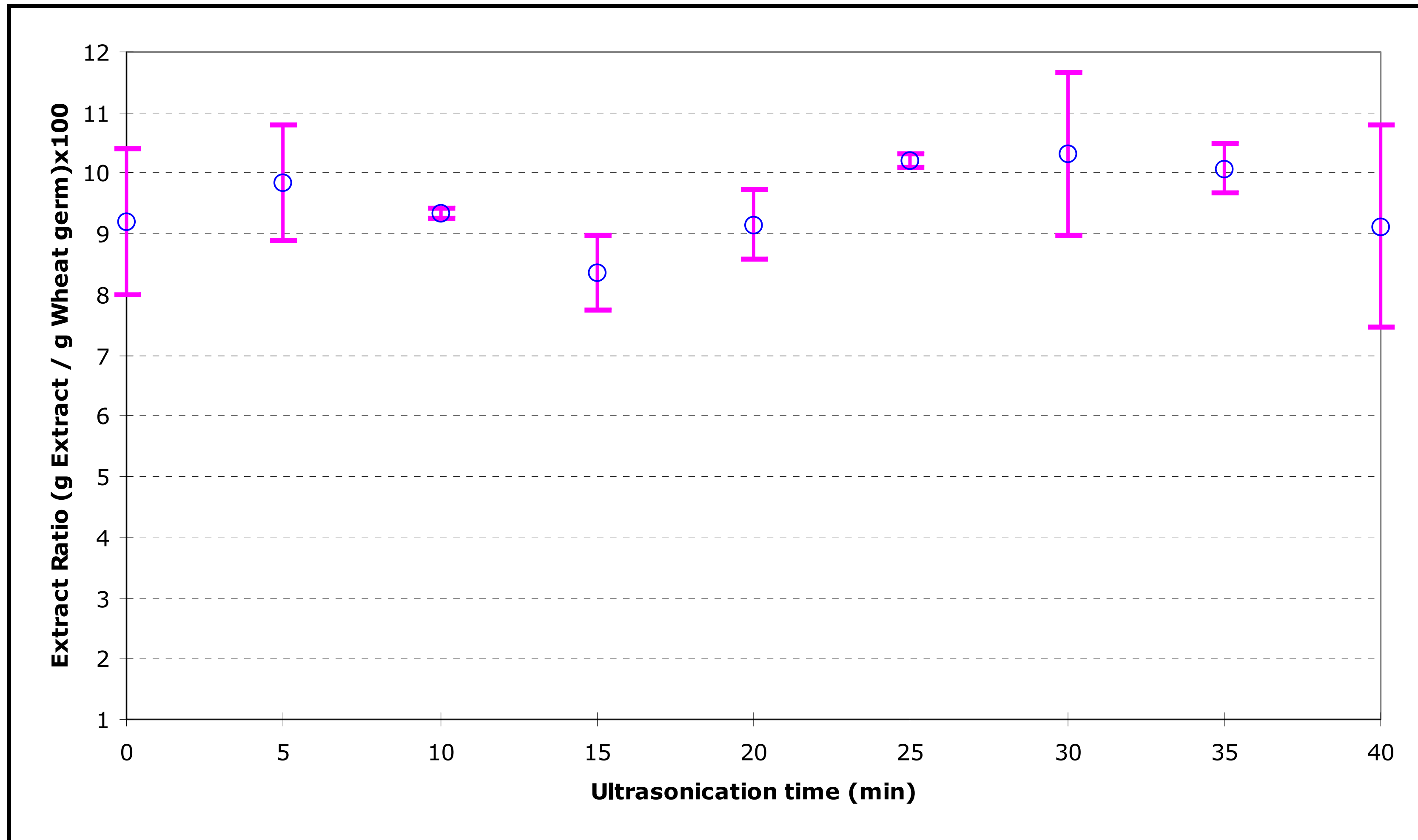

Figure 10 Extract Ratio vs. Ultrasonication time graph of ethanol

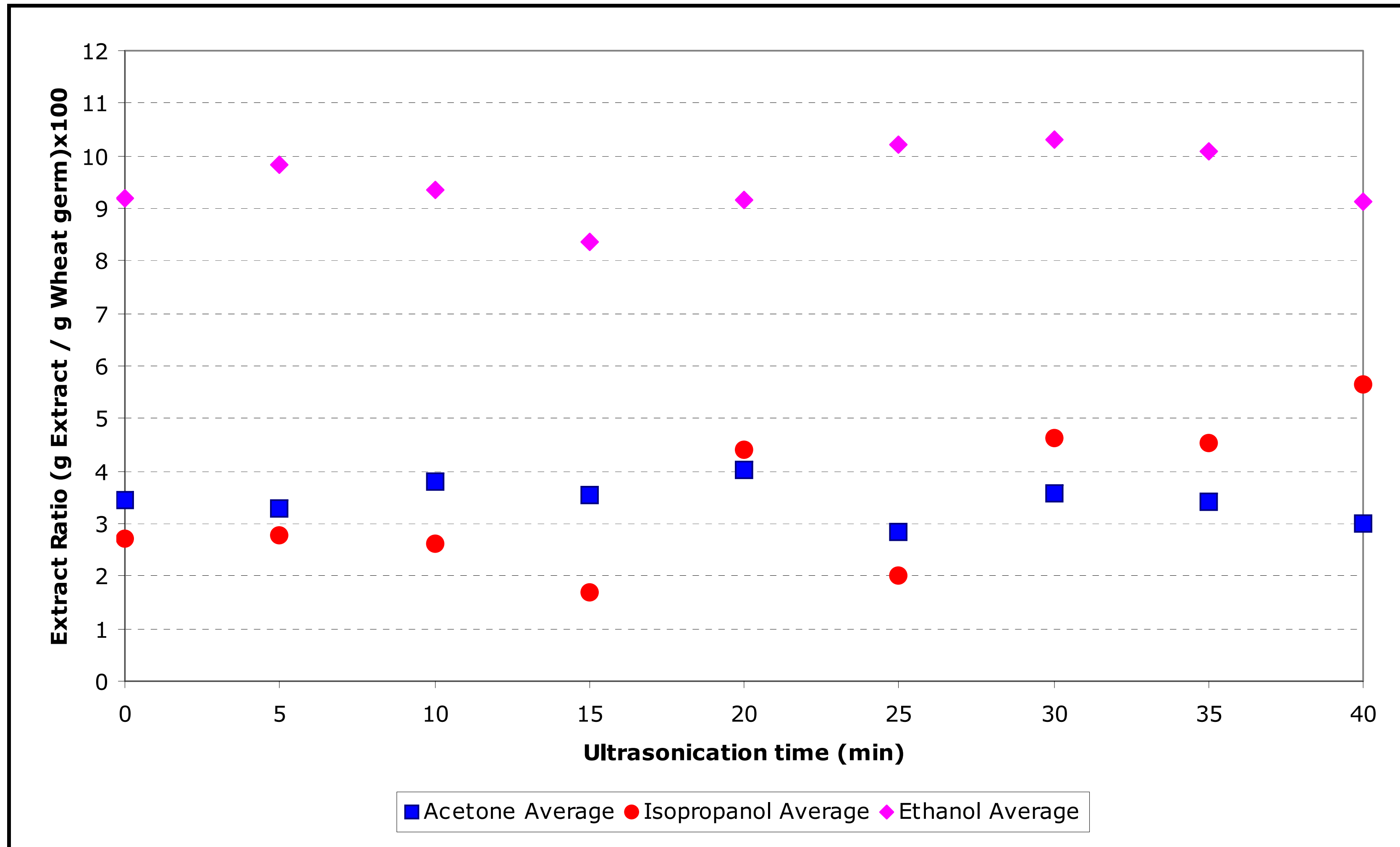

Figure 11 Average Extract Ratios vs. Ultrasonication time graph - All solvents

As it was seen form Table 18, for 25 minutes of sonication 99 % and for 5 minutes 95 %of the highest yield was achieved. Thus it was decided to use these points in the next part of the analysis.

Table 18 Comparison of the extract ratios

Sample	Time (min)	(g Extract / g Wheat germ) * 100	Ratio of each point to the maximum yield
0	0	9.2	0.89
1	5	9.8	0.95
2	10	9.3	0.91
3	15	8.4	0.81
4	20	9.2	0.89
5	25	10.2	0.99
6	**30**	**10.3**	**1.00**
7	35	10.1	0.98
8	40	9.1	0.89

4.4 ENHANCEMENTS IN THE SEPARATION OF THE SOLID AND THE LIQUID PHASES

The extraction procedure explained in the Appendix A4 was conducted for the specified parameters:

- Germ: AUSAS August 2004, nonroasted, roasted

- Solvent: 30 ml Ethanol
- Sonication time: 0, 5 and 25 min.

It was known that the best way to separate solid and liquid phases was centrifugation. Thus in order to enhance the phase separation and analyze its effects on the extract ratio, the effects of centrifugation and the duration inside the refrigerator were investigated. The procedures were given in the Section 3.8. The numbers in the x-axis of Graphs in Figures 12, 13 and 14 denotes these procedures. And the extract ratios were denoted as extract yields.

The effect of roasting on the extract yield was investigated briefly in this section. However the effect of roasting on the polyphenol extraction was investigated in great detail. The experimental data were given in the Appendix B4.

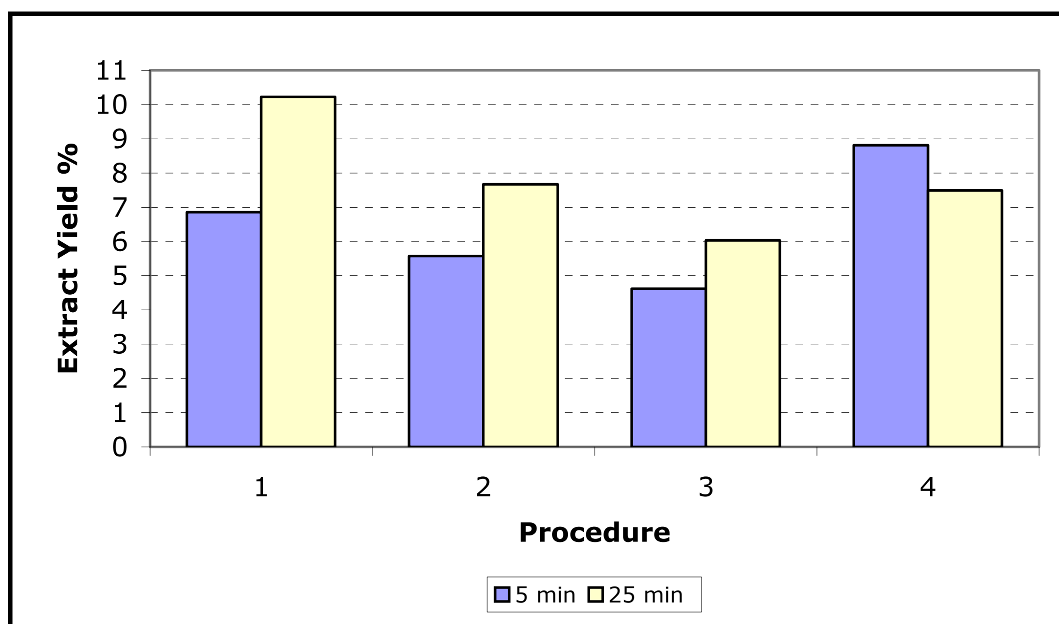

Figure 12 Separation of solid and liquid phases of nonroasted wheat germs

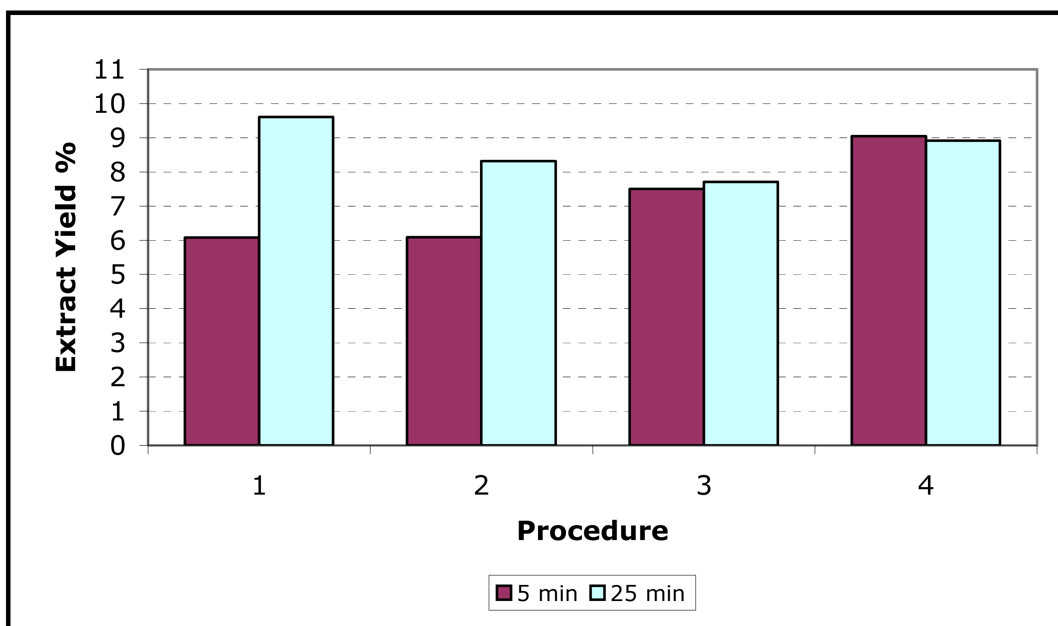

Figure 13 Separation of solid and liquid phases of roasted wheat germs

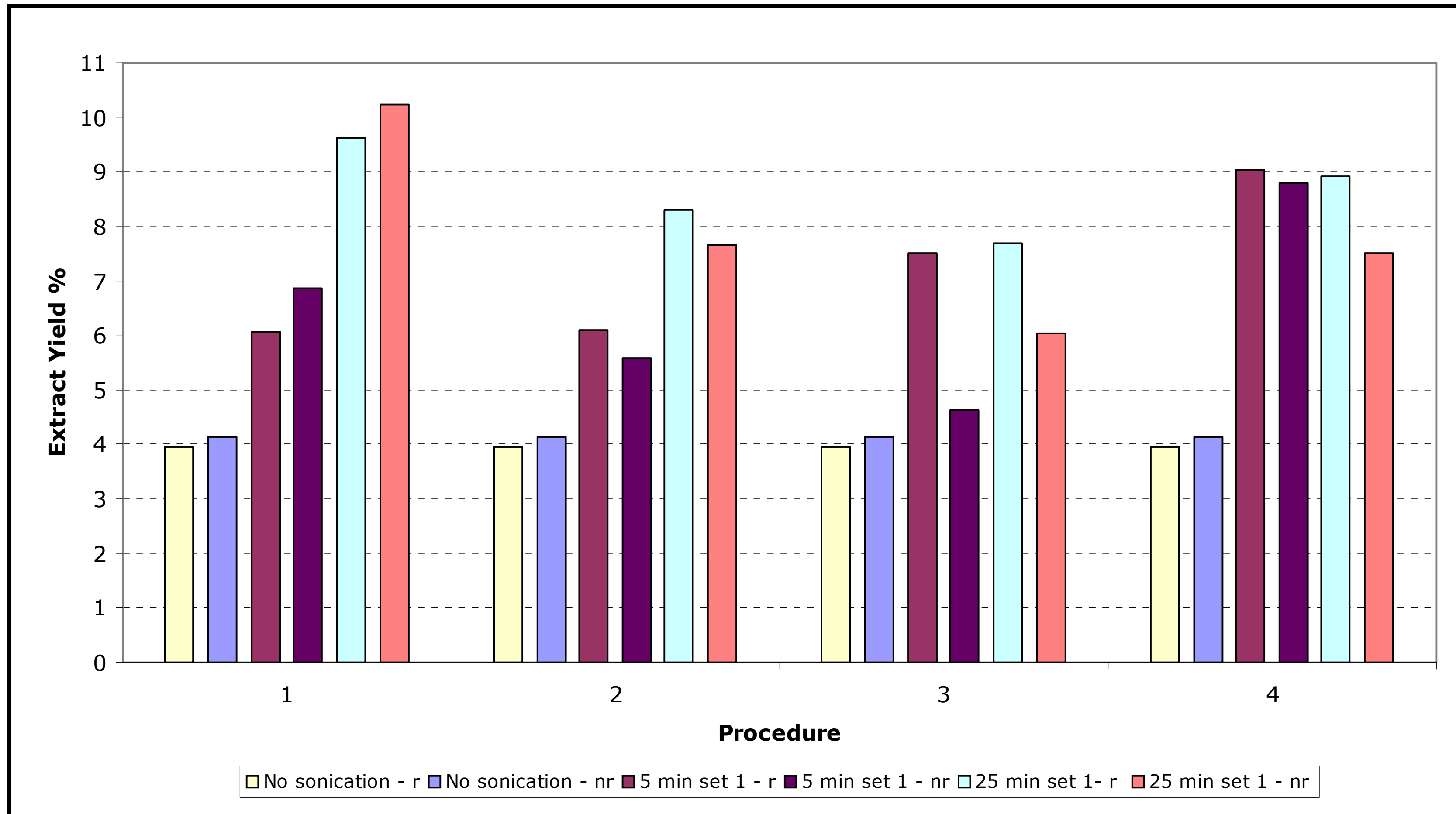

Figure 14 Separation of solid and liquid phases

As it was seen from Figure 14, the highest extract yield was obtained when Procedure 1 was conducted. In the Procedure 1 the extracts were stored at +4 º C in a refrigerator for 18-24 hours. Then the liquid phase was decanted. However as it was stated earlier some solid particles could remain in the liquid phase. Also from Figure 14 it was seen that the yield when Procedure 4 was carried out, is as high as Procedure 1.

In Procedure 4 the extracts were stored at +4 º C in a refrigerator for 18-24 hours. The liquid phase was gently poured into the centrifuge cartridge and centrifugation at 2800 rpm for 20 min. was conducted. Then the liquid phase was poured through a funnel using a filter paper into another beaker. In this procedure nearly no solid was left in the liquid phase thus any insoluble material was transferred to the liquid phase. So it was decided to carry out that Procedure 4 in the analysis of total polyphenol contents.

An interesting point that was encountered in this set of experiments was for all Procedures from 1-4 and for no sonication, 5 min and 25 min of sonication times there was an increasing trend in the extract ratios. Specifically for the Procedure 1 which was the same technique used for the extraction experiments tabulated in Section 4.3 there was a significant difference between the extraction patterns. As it is seen from Table 19, as the extract ratios increase with time for the experiments done in this section, the ratios for the previous sets of experiments were kept almost constant with an increase in time. The possible reason for this phenomenon was explained below.

The two sets of experiments were carried out using two different sets of wheat germs and for the experiments carried out in Section 4.3 it was seen

that for no sonication nearly the same amount of extract was collected for 25 min of sonication this can be caused due to the treatments in the milling plant as these germs were obtained by cracking and rolling, the germs were passed through lots of processes where high pressures were exerted on the surface of the germs thus these forces may damaged the cells on the surface. Thus when the germs were kept inside the solvents, by simple diffusion; the lipid phase was transferred into the solvent phase. This assumption is validated such that for no sonication the extract ratio was 9.2 which was % 90 of the highest ratio achieved in the sonication process. Meanwhile for the experiments carried out in this section the germs might not be as crashed as the germs in the previous section since the extract ratio for no sonication was only 4.1 where for 25 min the ratio was 10.2. So for wheat germs which are abraded in the milling processes some portion of the extract was transferred into the solvent phase without the effect of the sonication.

Table 19 Comparison of the average extract ratios

Time (min)	Average Extract Ratios	
	Section 4.3	**Section 4.4**
0	9.2	4.1
5	9.8	6.9
25	10.2	10.2

4.5 TOTAL POLYPHENOL CONTENT

The extraction procedure explained in the Appendix A4 was conducted for the specified parameters:

- Germ: AUSAS August 2004, nonroasted, roasted
- Solvent: Ethanol
- Germ to Solvent Ratio: 2 gram to 20 ml ethanol (1:10)
- Sonication time: 0, 5, 10, 15, 20, 25, 30, 35 and 40 minutes.

Then the solid and liquid phases were separated using the procedure given in the Procedure 4 of Section 3.8 and from each liquid phase the total polyphenol content was calculated applying the procedure given in the Appendix A7.

For roasted and nonroasted wheat germs linear regressions were tried to be found between the TPC and ultrasonication time. For nonroasted wheat germs, from the F –Test it was seen that $F = 23.0 > F_{CRITICAL}$ and from the LOF test it was seen that $F_{LOF} < F_{CRITICAL}$ which means lack of fit was insignificant. Thus there was no reason to doubt the adequacy of the model. For the roasted wheat germs from the F –Test it was seen that $F = 17.0 > F_{CRITICAL}$. However for the LOF test it was seen that F > F tabulated thus the lack of fit was significant so it is concluded that the linear regression was not significant. Triplicate experiments were carried and the experimental data and the statistical analysis were given in the Appendix B5. Total polyphenol contents of roasted and nonroasted wheat germs were given in Figures 15 and 16 respectively.

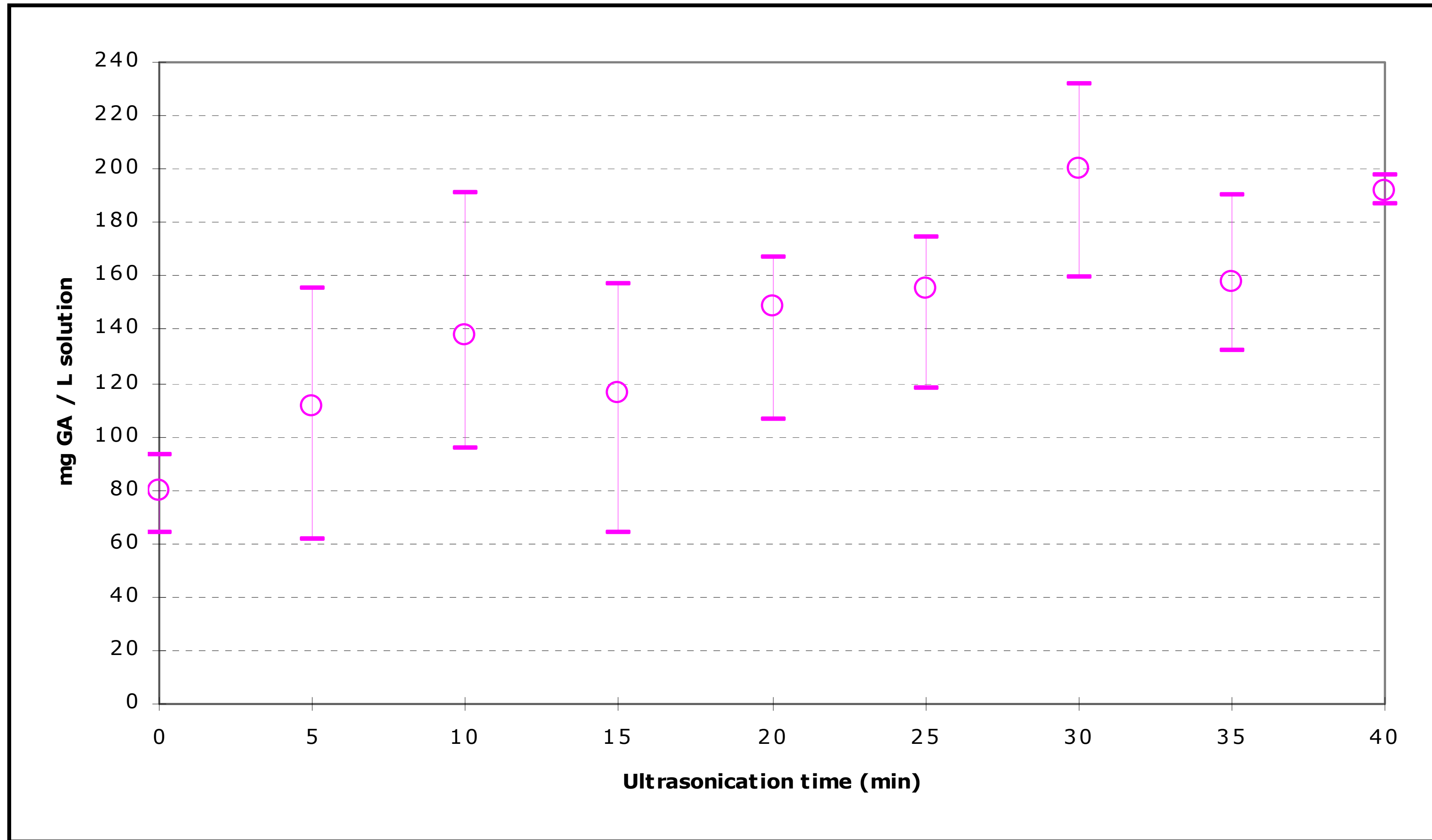

Figure 15 TPC of nonroasted wheat germs

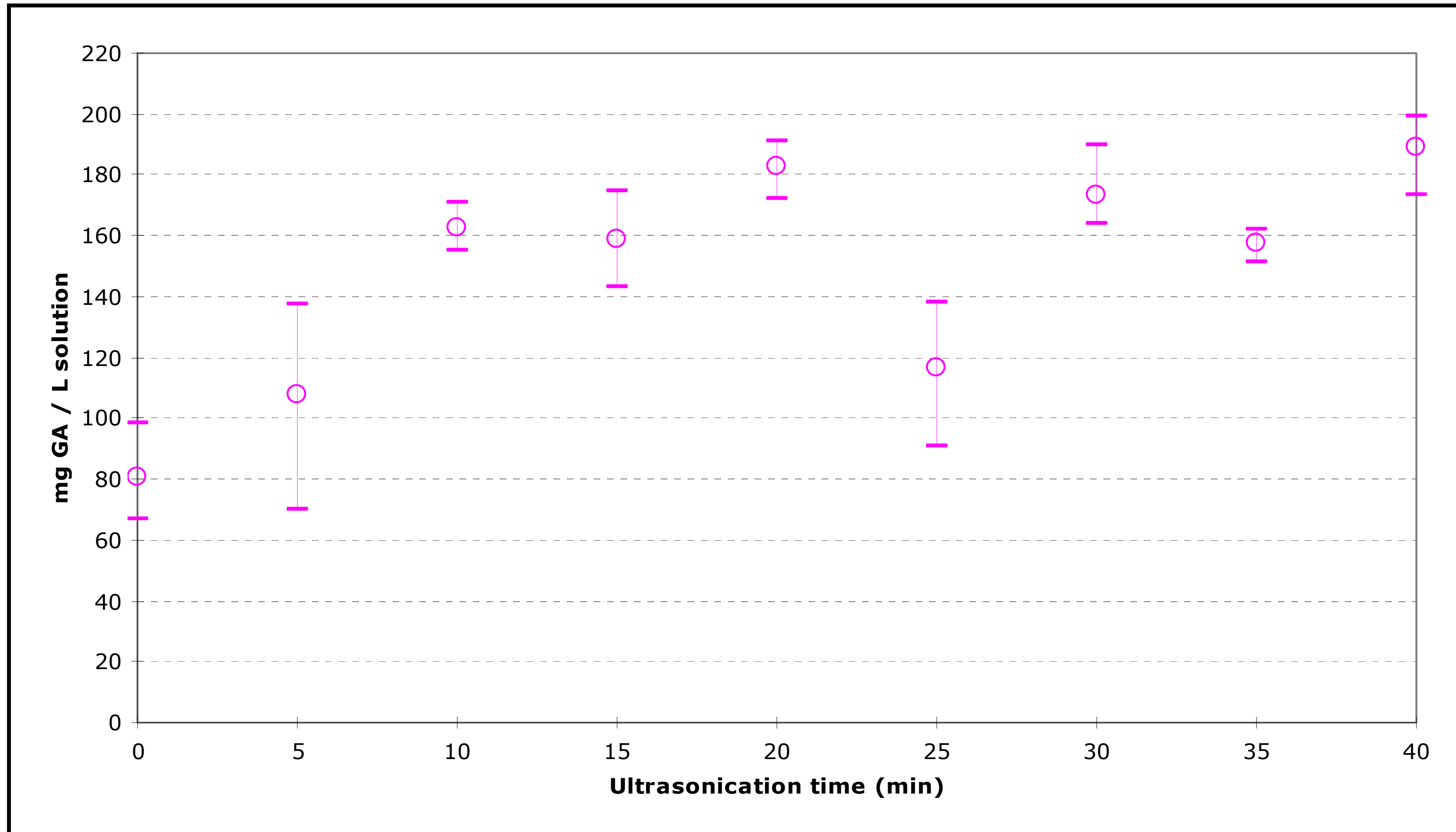

Figure 16 TPC of roasted wheat germs

From Figures 15 and 16 it was seen that for ultrasonic extraction of 40 minutes for both roasted and non roasted wheat germs the total polyphenol contents were approximately 190 mg GA / L solution.

CHAPTER 5

CONCLUSIONS

In this thesis work ultrasound assisted extraction of lipids and antioxidants from wheat germs were carried out. The wheat germs were characterized in terms of their extract yield and polyphenol contents.

Three alternative solvents were tested for the extraction of lipids and polyphenols from the wheat germ. The highest extract yield was achieved with ethanol. From the statistical analysis it was seen that the extract yields for ethanol and acetone were statistically insignificant thus there were no linear relation between the extract ratios and ultrasonication time. Mean of the extract ratios represent the data better than any other linear regression. However for isopropanol a linear relation was found between the extract ratio and ultrasonication time.

The separation of the solid and liquid phases after the extraction was also a great concern thus four methods were tested in order to find the best separation technique. It was observed that keeping the extracts in the refrigerator for 24 hours and then centrifugation gave the second highest yield but the best separation.

The extraction pattern for points; no sonication, 5 & 25 minutes, were different for extraction and separation enhancement experiments carried out with ethanol. The possible reason for that difference was the difference in the sets of germs that were used in the experiments. Also from no sonication experiments it was seen that there was a portion of extract that directly diffuses into the solvent matrix.

The total polyphenol contents of both the roasted and non roasted wheat germs were determined. The average polyphenol extracts were expressed in terms of mg gallic acid / L solution. For non roasted wheat germs significant linear regressions was found between total polyphenol contents of the extracts and ultrasonication time. However for the roasted wheat germs a linear regression can not be found. Total polyphenol contents of 200 mg GA / L solution was achievable with 30 minutes of ultrasonication for nonroasted wheat germs. Meanwhile, for roasted wheat germs 190 mg GA / L solution was achievable with 40 minutes of ultrasonication.

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APPENDIX A

PROCEDURES

A1 MOISTURE CONTENT

For the determination of the moisture content of the raw and roasted wheat germs the following procedure was used:

1. A watch glass and its lid were dried in the oven to constant weight.
2. Afterwards the watch glass was transferred to a dessicator to cool to the room temperature and its weight was recorded as w_{G1}.
3. Approximately 2 grams of germ was weighed, w_S, in this watch glass and placed into the oven at 105 º C.
4. The watch glass was kept in the oven for 2 hours.
5. After 2 hours the watch glass was covered with the lid.
6. The watch glass was placed into dessicator to cool to the room temperature and the final weight of the watch glass and its contents was recorded as w_{G2}.

The moisture content was calculated on the wet basis of raw and roasted wheat germs using Eqn.1.

$$Moisture\ Content, \% = \frac{w_S - (w_{G2} - w_{G1})}{w_S} * 100, \qquad \text{(Eqn.1)}$$

w_{G1}: weight of the empty watch glass, g

w_{G2}: weight of the watch glass and its contents, g

w_S: weight of the sample, wet basis, g

A2 ASH CONTENT

For the determination of the ash content of the raw and roasted wheat germs the following procedure was used:

1. Porcelain crucibles and lids are pre-heated around 600 º C overnight in the muffle furnace.
2. Afterwards the crucible was transferred to a dessicator to cool to the room temperature and its weight was recorded as w_{C1}.
3. Approximately 5 grams of germ was weighed, w_S, in this crucible and placed into the muffle furnace at 900 º C.
4. The crucible was kept in the oven for 4 hours.
5. After 4 hours the temperature in the muffle furnace was gradually decreased and crucible was transferred to the dessicator.
6. The crucible was covered with the lid and cooled to the room temperature.
7. The final weight of the crucible and its contents was recorded as w_{C2}.

The ash content is calculated on the wet basis of raw and roasted wheat germs of the party AUSAS August 2004 using Eqn.2.

$$Ash\ Content, \% = \frac{w_{C2} - w_{C1}}{w_S} * 100, \qquad \text{(Eqn.2)}$$

w_{C1}: weight of the empty crucible, g

w_{C2}: weight of the crucible and its contents, g

w_S: weight of the sample, wet basis, g

A3 FAT CONTENT

The fat content of the raw and roasted wheat germs was determined according to the AOCS Methods as follows:

1. The cellulose extraction cartridge of the soxhlet and an empty 300 ml beaker was dried in an oven.
2. Afterwards the beaker and the cartridge were transferred to a dessicator, to cool to the room temperature.
3. Approximately 5 grams of germ was weighed, w_S, and placed into cartridge.
4. The cartridge was placed inside the soxhlet flask.
5. The bottom part of the soxhlet is filled with n-hexane, approximately 130 ml.
6. The bottom part is placed into the electrical heater, the soxhlet flask was attached to the bottom part and finally the condenser was attached to the top. Thus a complete soxhlet extractor is prepared.
7. Then the cooling water was opened.
8. The extraction was carried out for 360 minutes.
9. The beaker inside the dessicator was taken out and the weight of the beaker was recorded as w_{B1}.

10. The solvent plus the fat collected in the bottom flask of the soxhlet was transferred to this beaker.
11. The solvent was vaporized under the hoods for 30 minutes at 110 º C.
12. The final weight of the beaker was recorded as w_{B2}.

The fat content is calculated on the wet basis of raw and roasted wheat germs using Eqn.3.

$$Fat\ Content, \% = \frac{(w_{B2} - w_{B1})}{w_S} * 100 , \qquad \text{(Eqn.3)}$$

w_{B1}: weight of the empty beaker, g

w_{B2}: weight of the beaker and its contents, g

w_S: weight of the sample, wet basis, g

A4 ULTRASONIC EXTRACTION

The main ultrasound assisted extraction procedure extraction procedure is as follows:

1. Erlenmeyer flasks with volumes 50 ml were cleaned and dried in the oven. And placed in the desiccators to cool to the room temperature.
2. Specific amount of wheat germs are weighed inside these flasks, w_S.
3. Specific amount of solvents, v_S, were added to the flasks. And the tops of the flasks are closed with polyethylene films.
4. The ultrasonic bath was filled with distilled water, 4 cm below the top, shown in Figure 17.

5. Flasks are placed inside the bath such that the solvent inside the flasks are 2 cm below the surface of the water.
6. The sonication is carried out for time, t_S.
7. The flasks are taken out the bath cleaned and placed into the refrigerator.

w_S: weight of the sample, g

v_S: volume of the solvent, ml

t_S: time of sonication, min.

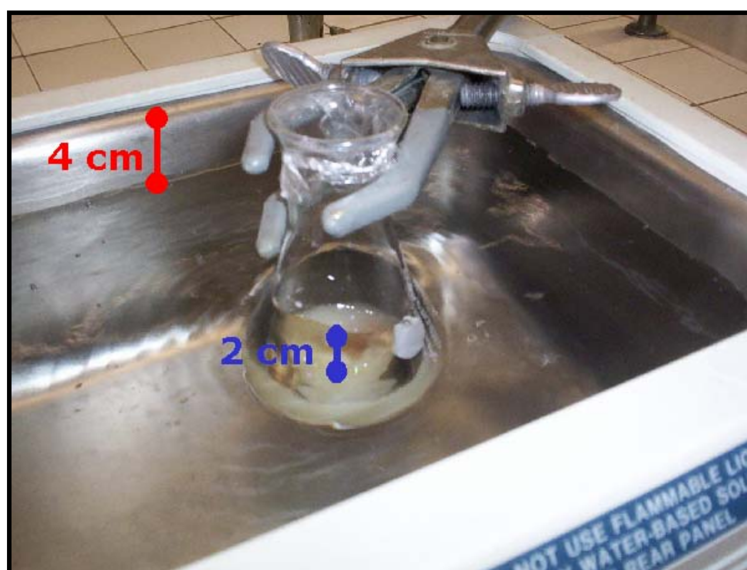

Figure 17 Positioning of the flask inside the ultrasonic bath

A5 DECANTATION

The following modified way of decantation was used:

1. The extracts obtained are stored at +4 º C in a refrigerator for 18-24 hours in order to facilitate the phase separation.

2. For decanting another erlen or beaker is used. Beakers with volumes 100 ml were cleaned and dried in the oven. And placed in the desiccators to cool to the room temperature, where the weight of the beaker was recorded as w_{B1}.
3. The solution is poured from the erlenmeyer flasks, leaving the precipitate (solid phase) in the bottom, through a funnel using a filter paper. Usually a small amount of solution is left in the erlen in order to prevent a small amount of precipitate from flowing with the solution out of the other beaker.

A6 SOLVENT EVAPORATION AND EXTRACT RATIO

The following procedure was used to evaporate the solvents:

1. Beakers with volumes 100 ml were cleaned and dried in the oven. And placed in the desiccators to cool to the room temperature.
2. The beaker inside the dessicator was taken out and the weight of the beaker was recorded as w_{B1}.
3. The solvent plus the fat collected after the further treatment was transferred to this beaker.
4. The solvent was vaporized under the hoods for 30 minutes at 110 º C.
5. The final weight of the beaker was recorded as w_{B2}.

The extract ratio is calculated on the wet basis of raw and roasted wheat germs using Eqn.4.

$$Extract\ Ratio, \% = \frac{(w_{B2} - w_{B1})}{w_S} * 100 , \qquad \text{(Eqn.4)}$$

w_{B1}: weight of the empty beaker, g

w_{B2}: weight of the beaker and its contents, g

w_S: weight of the sample, wet basis, g

A7 TOTAL POLYPHENOL CONTENT

The following procedure was used to determine the total polyphenol content which was adopted from the work done by **Waterhouse, A.** and **Yu et al. 2002** and **Yu et al., 2003**.

First the gallic acid and the Sodium Carbonate Stock Solutions were prepared.

Gallic Acid Stock Solution: In a 100 mL volumetric flask, 0.500 g of dry gallic acid is dissolved in 10 mL of ethanol and diluted with distilled water.

Sodium Carbonate Solution: 50 g of anhydrous sodium carbonate is dissolved in 200 mL of water via heated on a magnetic stirrer until the water boils. After cooling, a few crystals of sodium carbonate were added to the solution. The solution was kept for 24 hr then filtered and distilled water was added to bring the total volume 250 ml. This will make a 20% sodium carbonate solution.

Then the calibration curve is prepared using the gallic acid (phenol) stock solution. In order to prepare the calibration curve,

1. Add 0, 1, 2, 3, 5, and 10 mL of the above gallic acid stock solution into 100 mL beakers, and dilute with distilled water.

2. These solutions will have phenol concentrations of 0, 50, 100, 150, 250, and 500 mg/L gallic acid.

From each calibration solution, sample, or blank:

1. 0.2 ml was taken into separate beaker,
2. Then 1 ml of the Folin-Ciocalteu's phenol reagent (Sigma) and 3 ml of Sodium Carbonate Solution were added.
3. The sample was diluted to 20 ml with distilled water.
4. After 2 hours of reaction time at room temperature or 30 min at 40 º C the absorbance of each solution is read at *766.4 nm* (instead of 765 nm) against the blank using the UV – Spectrophotometer, Hitachi U -3200.
5. The absorbance data was transferred into concentration data using the calibration curve given in Figure 18.

However each spectrophotometer the wavelength must be traced in order to check whether the predefined value reads the maximum absorbance or not. A test was carried out between 700 – 800 nm to see whether 765 nm gives the highest absorbance for 150 mg/L gallic acid calibration solution. The data of this analysis between 760 –770 nm is given on Table 20 and the plot from the UV Spectrophotometer for the whole range was given in the Appendix C. And the ultrasonic extracts were kept in dark between the analysis until the TPC was read.

Table 20 Wavelength check for 150 mg/L gallic acid calibration solution

Wavelength (nm)	**Absorbance**
760.0	0.3979
765.0	0.4024
766.4	**0.4200**
767.3	0.4055

From this analysis it was seen that *766.4 nm* gives the highest value for the absorbance, 0.42 so for the rest of the experiments absorbance were read at **766.4 nm** instead of 765 nm.

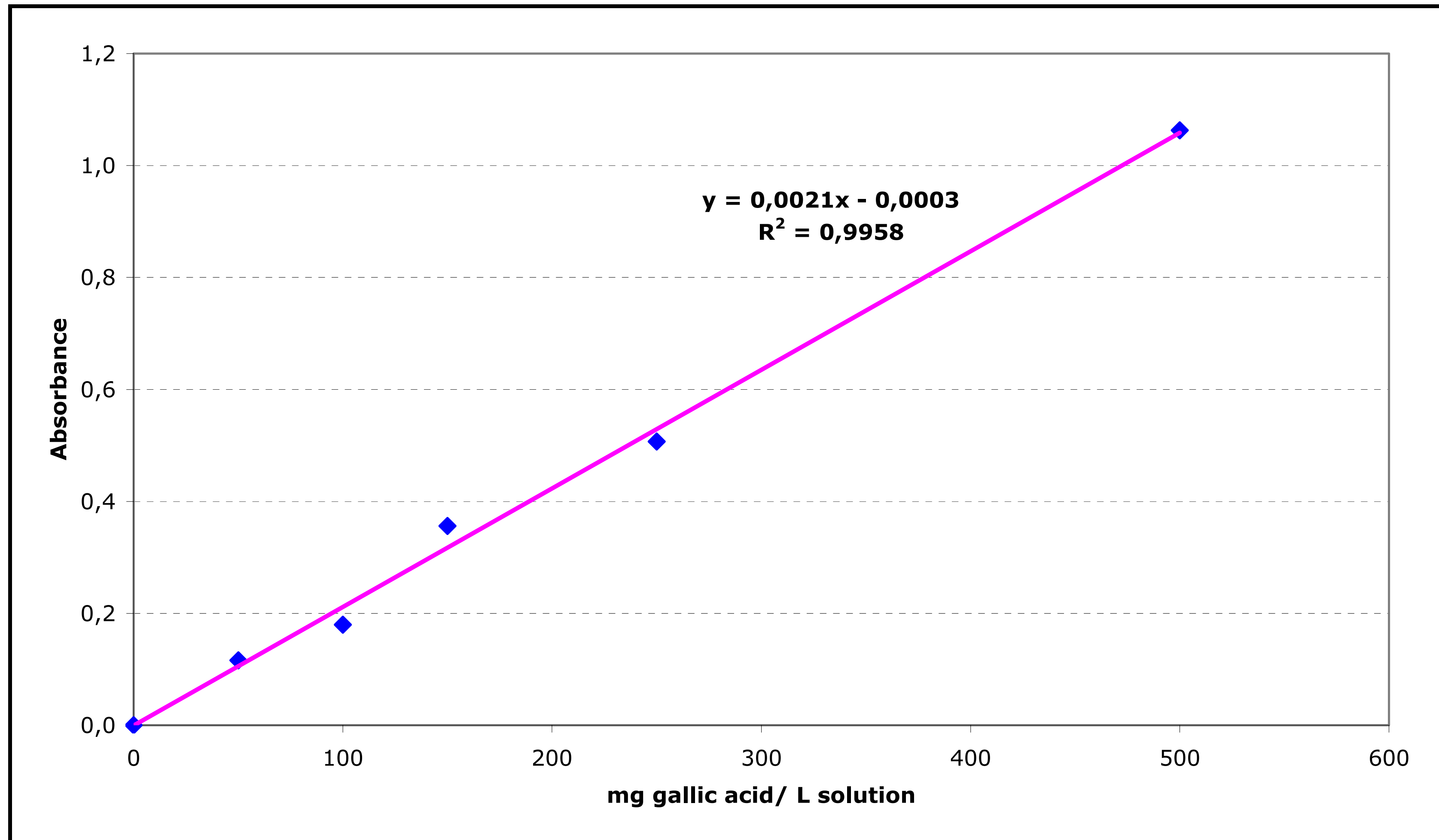

Figure 18 Gallic Acid Standard Curve

APPENDIX B

EXPERIMENTAL DATA

B1 EXTRACTION WITH ACETONE

Table 21 Ultrasonic extraction with acetone set 1

Sample	Time (min)	Solvent (ml)	Wheat Germ (g)	(g Extract / g germ) x 100
0	0	20.4	1.02	2.63
1	5	21.5	1.07	3.19
2	10	21.8	1.09	3.69
3	15	22.8	1.14	3.92
4	20	19.9	0.99	2.93
5	25	21.0	1.05	3.18
6	30	22.5	1.12	3.82
7	35	20.0	1.00	2.53
8	40	22.3	1.11	3.16

Table 22 Ultrasonic extraction with acetone set 2

Sample	Time (min)	Solvent (ml)	Wheat Germ (g)	(g Extract / g germ) x 100
0	0	24.3	1.21	4.29
1	5	24.9	1.24	3.39
2	10	25.3	1.26	3.88
3	15	22.0	1.10	3.19
4	20	21.0	1.05	5.12
5	25	25.1	1.26	2.49
6	30	24.1	1.21	3.29
7	35	21.8	1.09	4.29
8	40	22.2	1.11	2.84

Table 23 Ultrasonic extraction with acetone - average

Sample	Time (min)	Average (g Extract / g germ) x 100
0	0	3.46
1	5	3.29
2	10	3.78
3	15	3.55
4	20	4.03
5	25	2.83
6	30	3.56
7	35	3.41
8	40	3.00

The output of the data analysis program Microsoft Excel Analysis ToolPak was given on the next page.

B1.1 STATISTICAL ANALYSIS OF ACETONE EXTRACTS

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.17
R Square	0.03
Adjusted R Square	-0.03
Standard Error	0.71
Observations	18

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.23	0.23	0.47	5.04E-01
Residual	16	7.97	0.50		
Total	17	8.21			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 99.9%*	*Upper 99.9%*
Intercept	3.61	0.31	11.77	0.00	2.96	4.26	2.38	4.84
X Variable 1	-0.01	0.01	-0.68	0.50	-0.04	0.02	-0.06	0.04

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*
1	3.61	-0.98
1	3.61	0.67
2	3.57	-0.38
2	3.57	-0.17
3	3.52	0.16
3	3.52	0.36
4	3.48	0.44
4	3.48	-0.29
5	3.44	-0.51
5	3.44	1.69
6	3.39	-0.22
6	3.39	-0.90
7	3.35	0.48
7	3.35	-0.05
8	3.30	-0.77
8	3.30	0.99
9	3.26	-0.10
9	3.26	-0.42

From the F test it is found that $F < F_{CRITICAL}$ thus the **linear regression is insignificant**. Thus the mean represents the data better then any other linear regression.

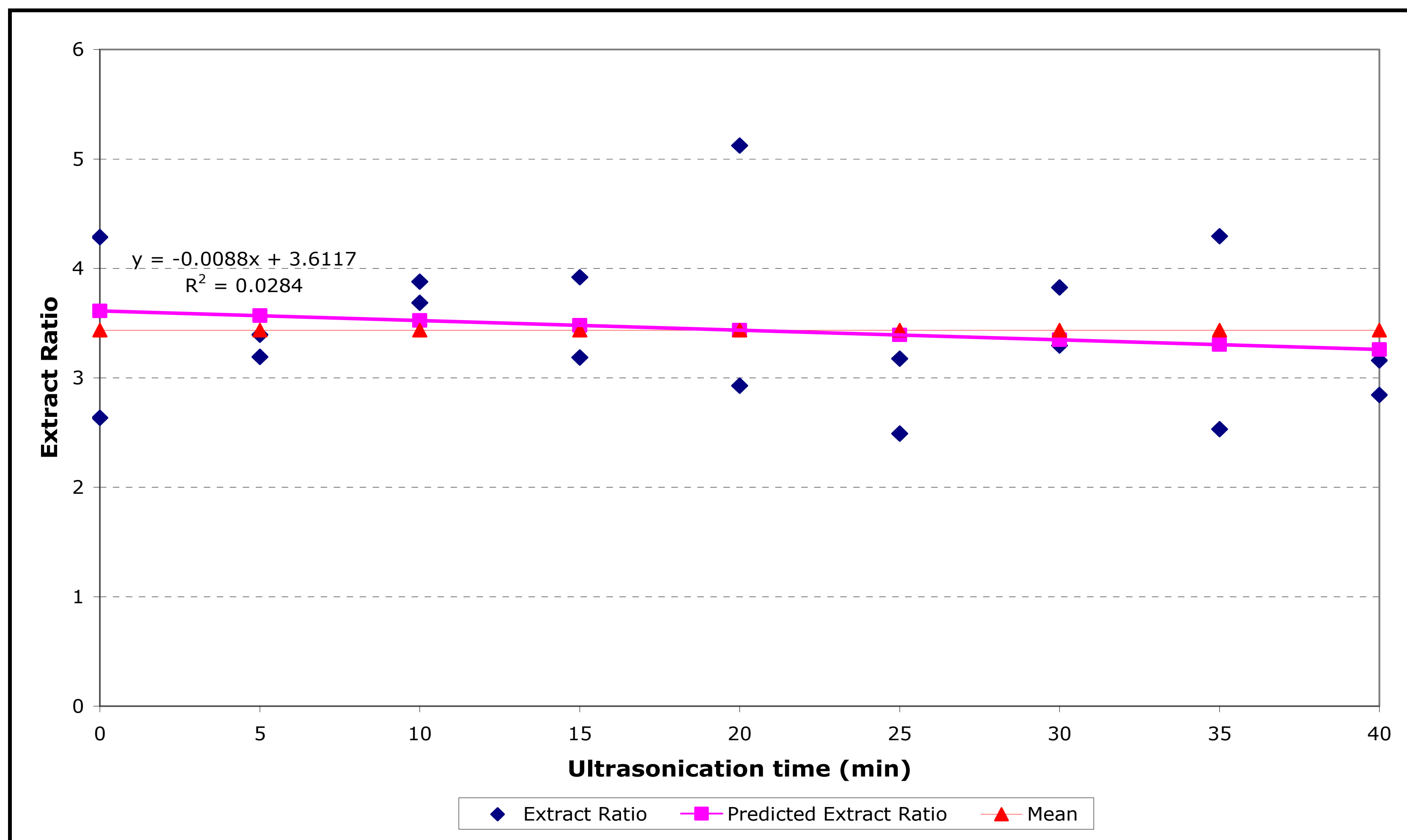

Figure 19 Extract Ratio vs. Ultrasonication time graph of Acetone - Model

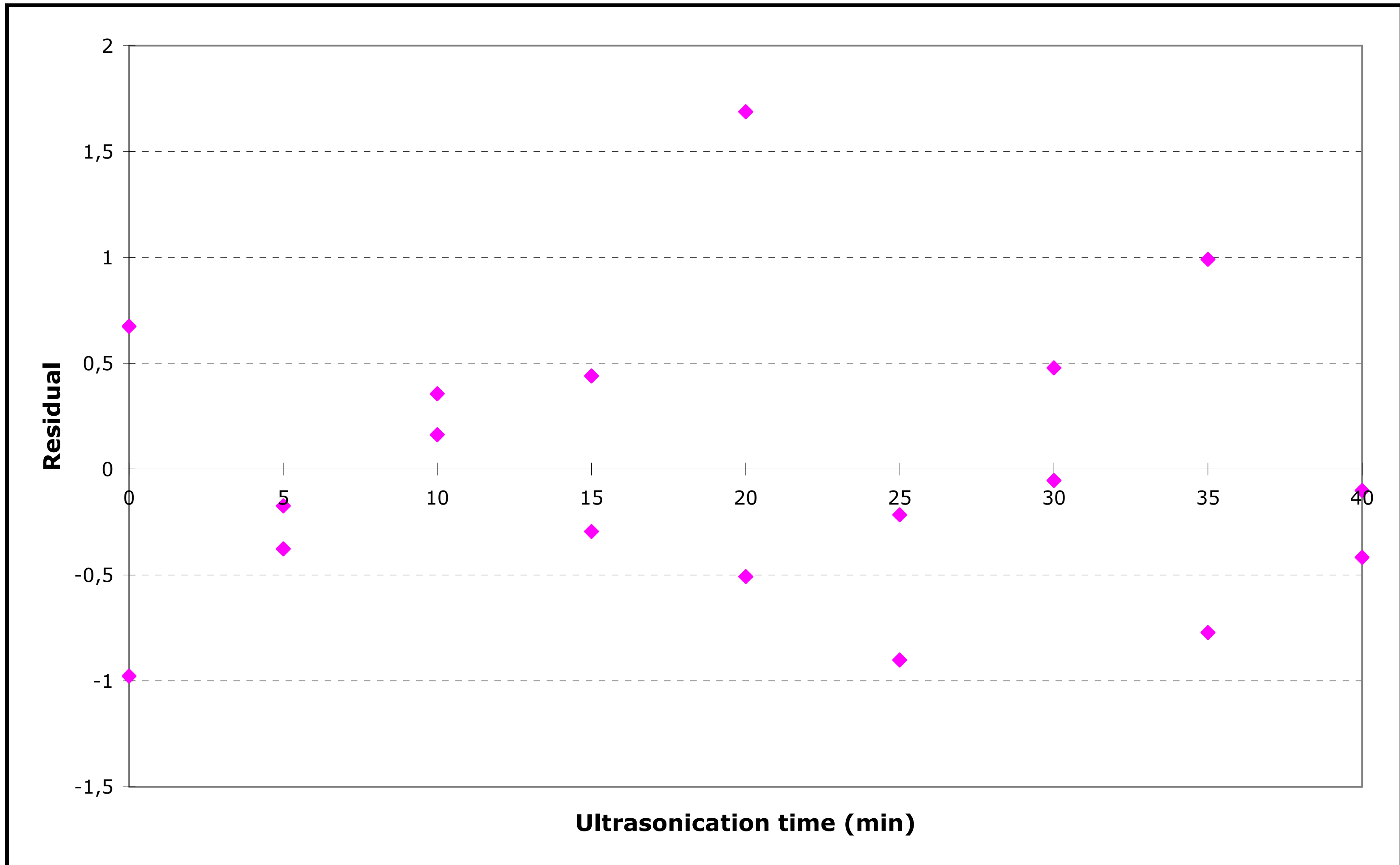

Figure 20 Residual Plot for Acetone Extracts

B2 EXTRACTION WITH ISOPROPANOL

Table 24 Ultrasonic extraction with isopropanol set 1

Sample	Time (min)	Solvent (ml)	Wheat Germ (g)	(g Extract / g germ) x 100
0	0	20.6	1.03	1.16
1	5	20.8	1.04	1.04
2	10	20.5	1.03	0.83
3	15	21.8	1.09	1.17
4	20	21.6	1.08	2.67
5	25	20.7	1.04	1.91
6	30	20.3	1.01	3.75
7	35	20.6	1.03	5.56
8	40	22.9	1.14	6.60

Table 25 Ultrasonic extraction with isopropanol set 2

Sample	Time (min)	Solvent (ml)	Wheat Germ (g)	(g Extract / g germ) x 100
0	0	22.4	1.12	4.23
1	5	22.6	1.13	4.52
2	10	23.8	1.19	4.40
3	15	20.8	1.04	2.20
4	20	21.0	1.05	6.14
5	25	22.5	1.13	2.12
6	30	25.7	1.29	5.48
7	35	23.1	1.15	3.49
8	40	22.3	1.12	4.68

Table 26 Ultrasonic extraction with isopropanol - average

Sample	Time (min)	Average (g Extract / g germ) x 100
0	0	2.70
1	5	2.78
2	10	2.61
3	15	1.68
4	20	4.40
5	25	2.02
6	30	4.62
7	35	4.52
8	40	5.64

The output of the data analysis program Microsoft Excel Analysis ToolPak was given on the next page.

B2.1 STATISTICAL ANALYSIS OF ISOPROPANOL EXTRACTS

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.51
R Square	0.26
Adjusted R Square	0.21
Standard Error	1.66
Observations	18

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	15.21	15.21	5.50	3.23E-02
Residual	16	44.27	2.77		
Total	17	59.48			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 99.9%*	*Upper 99.9%*
Intercept	2.02	0.72	2.79	0.01	0.49	3.55	-0.88	4.92
X Variable 1	0.07	0.03	2.34	0.03	0.01	0.14	-0.05	0.19

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	Pure Error Squares
1	2.02	-0.86	4.71
2	2.02	2.21	
3	2.37	-1.34	6.06
4	2.37	2.15	
5	2.73	-1.90	6.38
6	2.73	1.67	
7	3.09	-1.92	0.54
8	3.09	-0.88	
9	3.44	-0.78	6.04
10	3.44	2.70	
11	3.80	-1.89	0.02
12	3.80	-1.68	
13	4.15	-0.40	1.50
14	4.15	1.33	
15	4.51	1.05	2.14
16	4.51	-1.02	
17	4.87	1.74	1.84
18	4.87	-0.18	
			Sum 29.23

	df	SS	MS	F Ratio
LOF	7	15.04	2.15	0.66
Pure Error	9	29.23	3.25	Lack of Fit insignificant

From the F test it is found that $F > F_{CRITICAL}$ and

From the lack of fit test it is found that lack of fit was insignificant,

Therefore there is no reason to doubt the adequacy of the model based on these tests, which means the **linear regression is significant**.

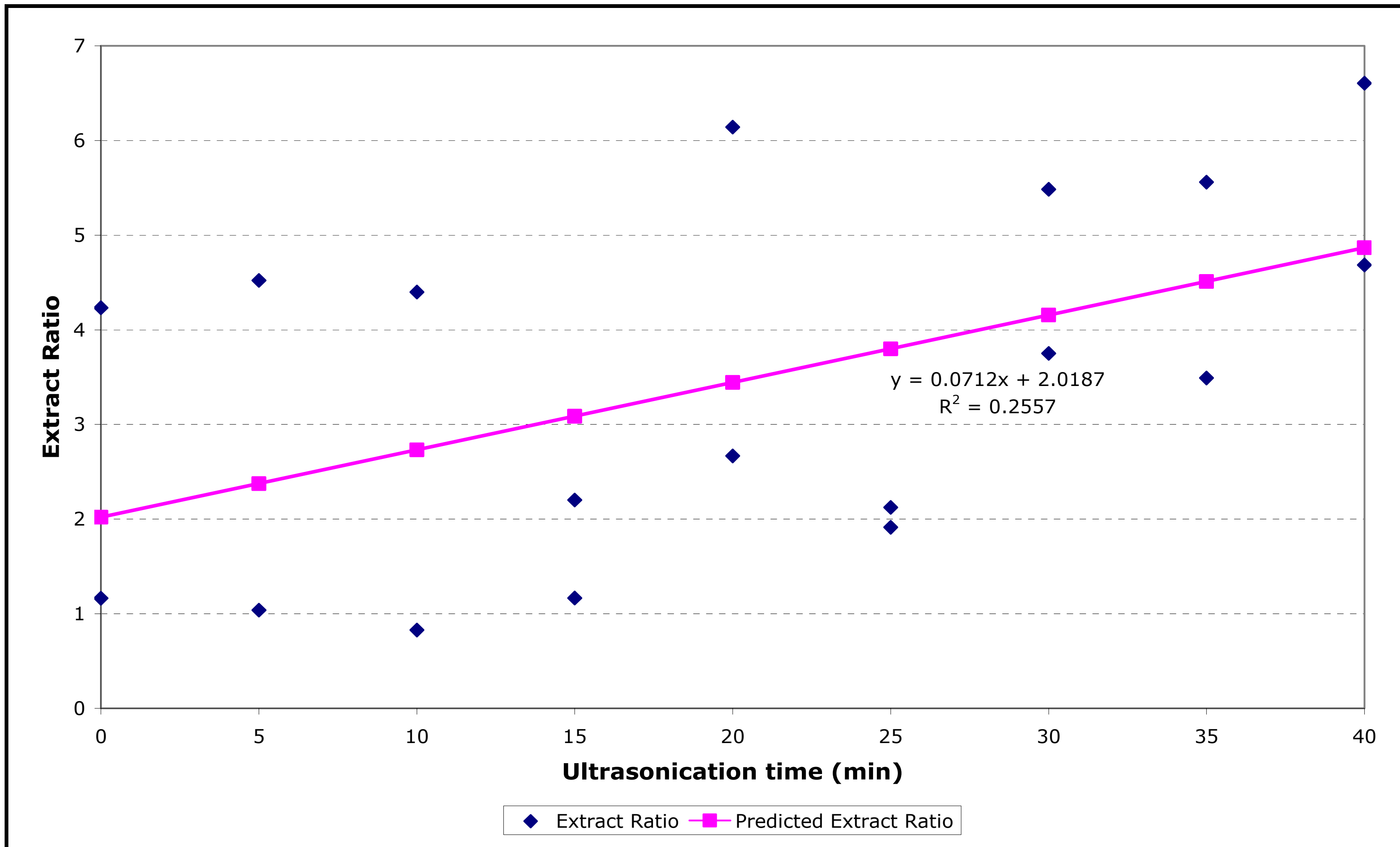

Figure 21 Extract Ratio vs. Ultrasonication time graph of Isopropanol - Model

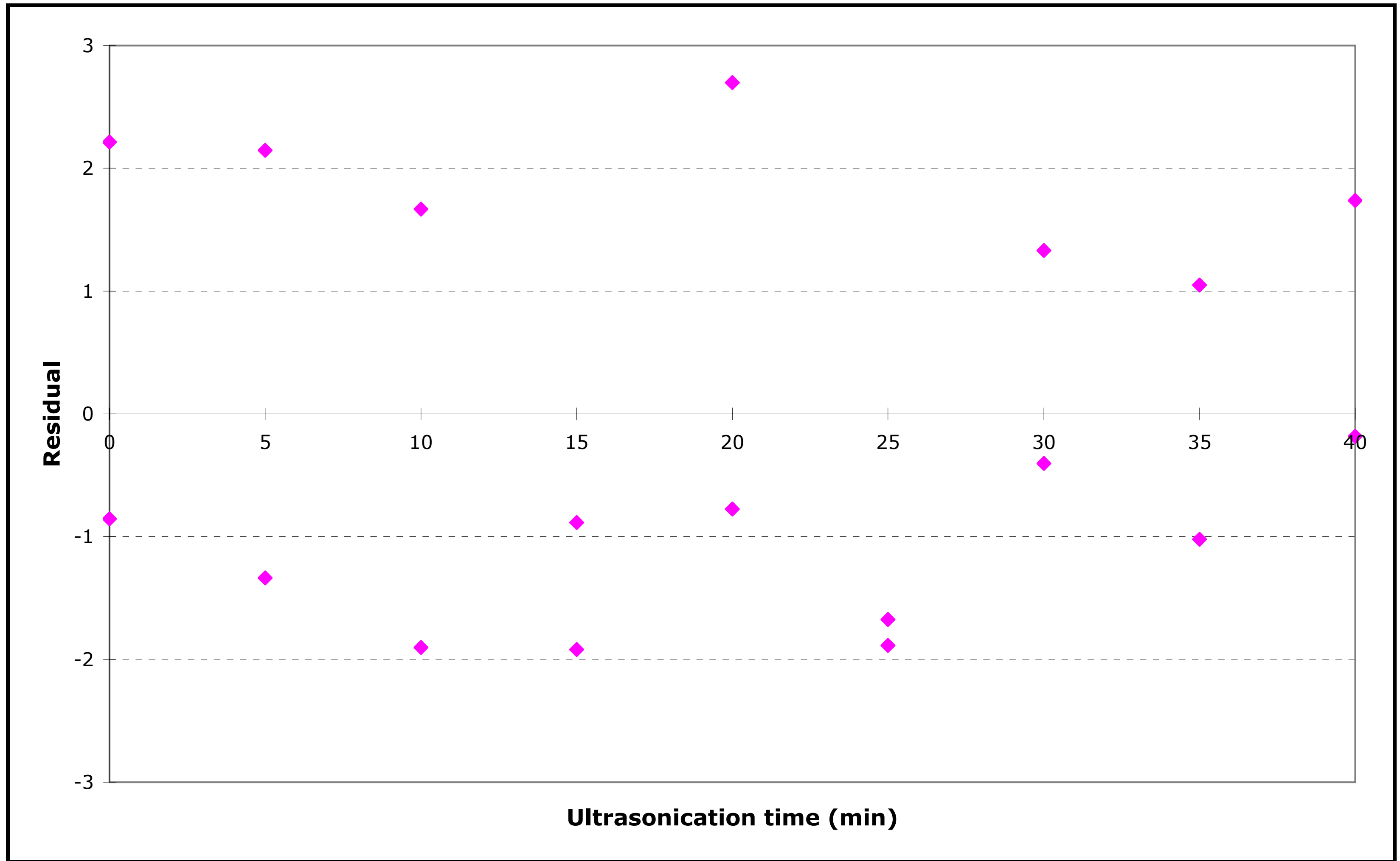

Figure 22 Residual Plot for Isopropanol Extracts

B3 EXTRACTION WITH ETHANOL

Table 27 Ultrasonic extraction with ethanol set 1

Sample	Time (min)	Solvent (ml)	Wheat Germ (g)	(g Extract / g germ) x 100
0	0	22.5	1.13	8.00
1	5	21.2	1.06	8.88
2	10	22.3	1.11	9.44
3	15	20.3	1.01	8.98
4	20	21.2	1.06	9.73
5	25	21.2	1.06	10.10
6	30	20.5	1.02	11.66
7	35	20.5	1.02	10.49
8	40	20.2	1.01	10.79

Table 28 Ultrasonic extraction with ethanol set 2

Sample	Time (min)	Solvent (ml)	Wheat Germ (g)	(g Extract / g germ) x 100
0	0	21.1	1.06	10.41
1	5	22.1	1.10	10.79
2	10	24.6	1.23	9.25
3	15	24.6	1.23	7.73
4	20	24.7	1.23	8.59
5	25	22.6	1.13	10.33
6	30	21.1	1.06	8.97
7	35	20.2	1.01	9.67
8	40	23.7	1.18	7.47

Table 29 Ultrasonic extraction with ethanol - average

Sample	Time (min)	Average (g Extract / g germ) x 100
0	0	9.21
1	5	9.83
2	10	9.34
3	15	8.36
4	20	9.16
5	25	10.22
6	30	10.31
7	35	10.08
8	40	9.13

The output of the data analysis program Microsoft Excel Analysis ToolPak was given on the next page.

B3.1 STATISTICAL ANALYSIS OF ETHANOL EXTRACTS

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.16
R Square	0.03
Adjusted R Square	-0.03
Standard Error	1.16
Observations	18

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.60	0.60	0.44	5.16E-01
Residual	16	21.69	1.36		
Total	17	22.29			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 99.9%*	*Upper 99.9%*
Intercept	9.23	0.51	18.25	0.00	8.16	10.31	7.20	11.26
X Variable 1	0.01	0.02	0.66	0.52	-0.03	0.06	-0.07	0.10

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*
1	9.23	-1.23
1	9.23	1.18
2	9.30	-0.42
2	9.30	1.48
3	9.37	0.06
3	9.37	-0.12
4	9.44	-0.47
4	9.44	-1.71
5	9.51	0.21
5	9.51	-0.93
6	9.59	0.51
6	9.59	0.75
7	9.66	2.00
7	9.66	-0.69
8	9.73	0.76
8	9.73	-0.05
9	9.80	0.99
9	9.80	-2.33

From the F test it is found that $F < F_{CRITICAL}$ thus the **linear regression is insignificant**. Thus the mean represents the data better then any other linear regression.

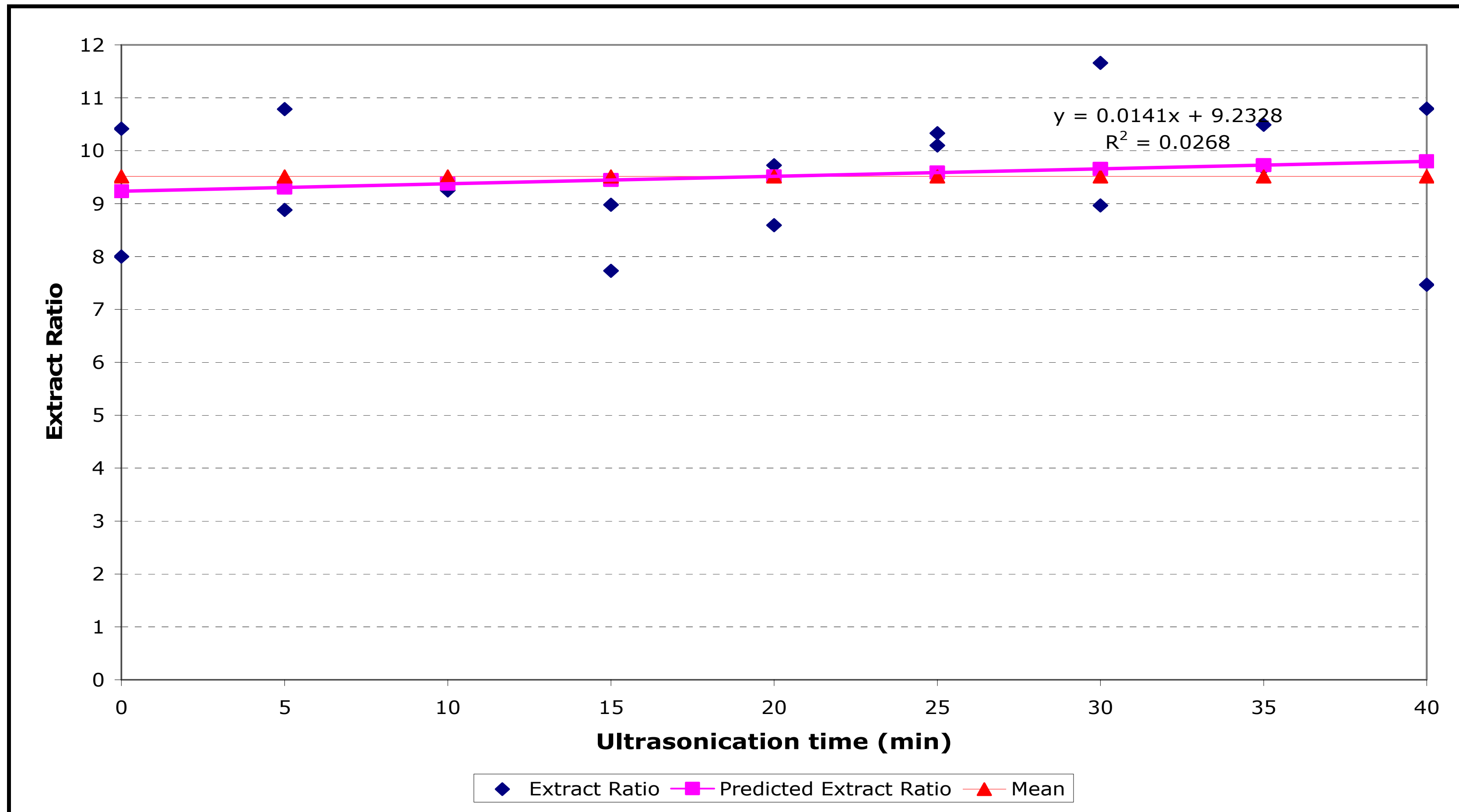

Figure 23 Extract Ratio vs. Ultrasonication time graph for Ethanol - Model

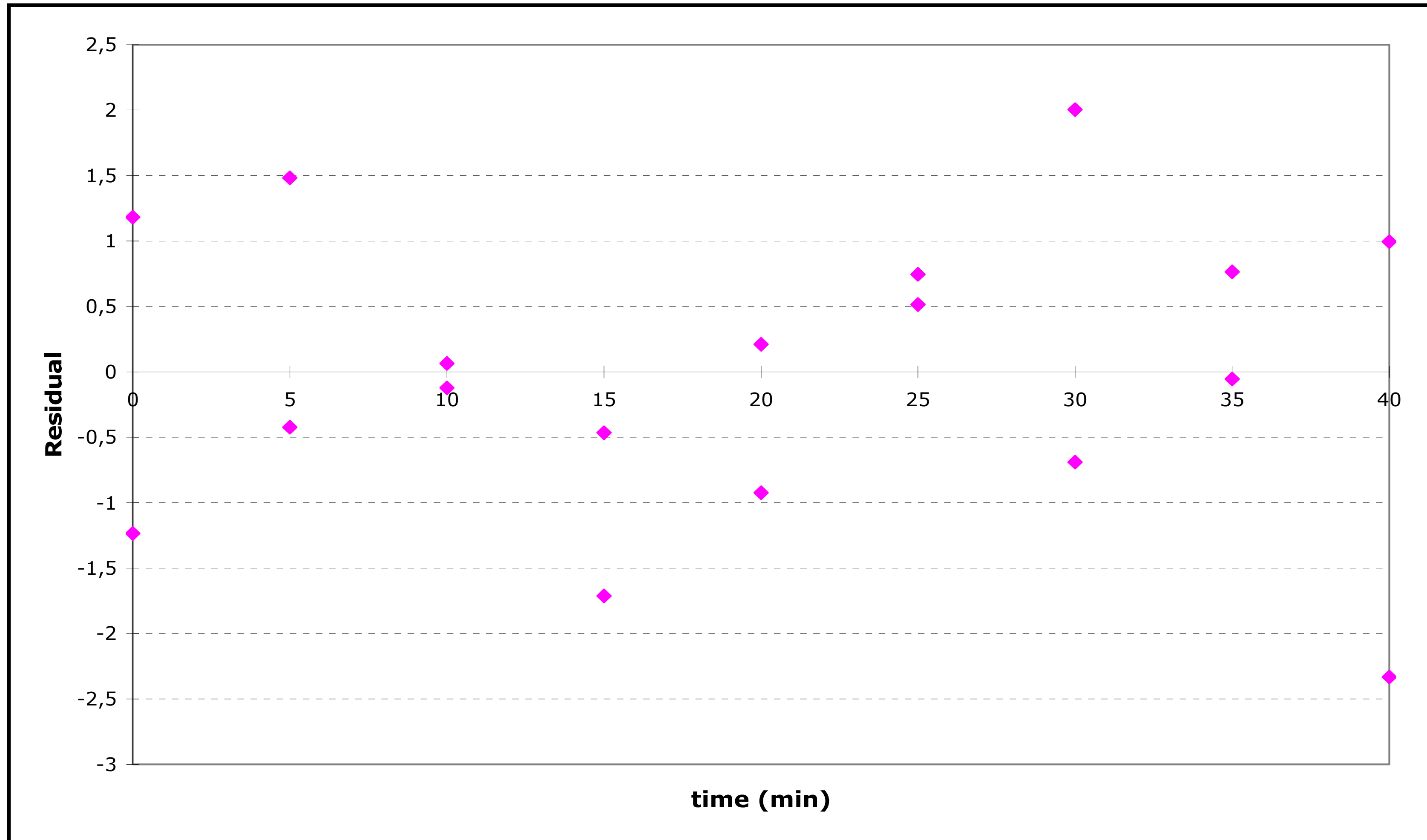

Figure 24 Residual Plot for Ethanol Extracts

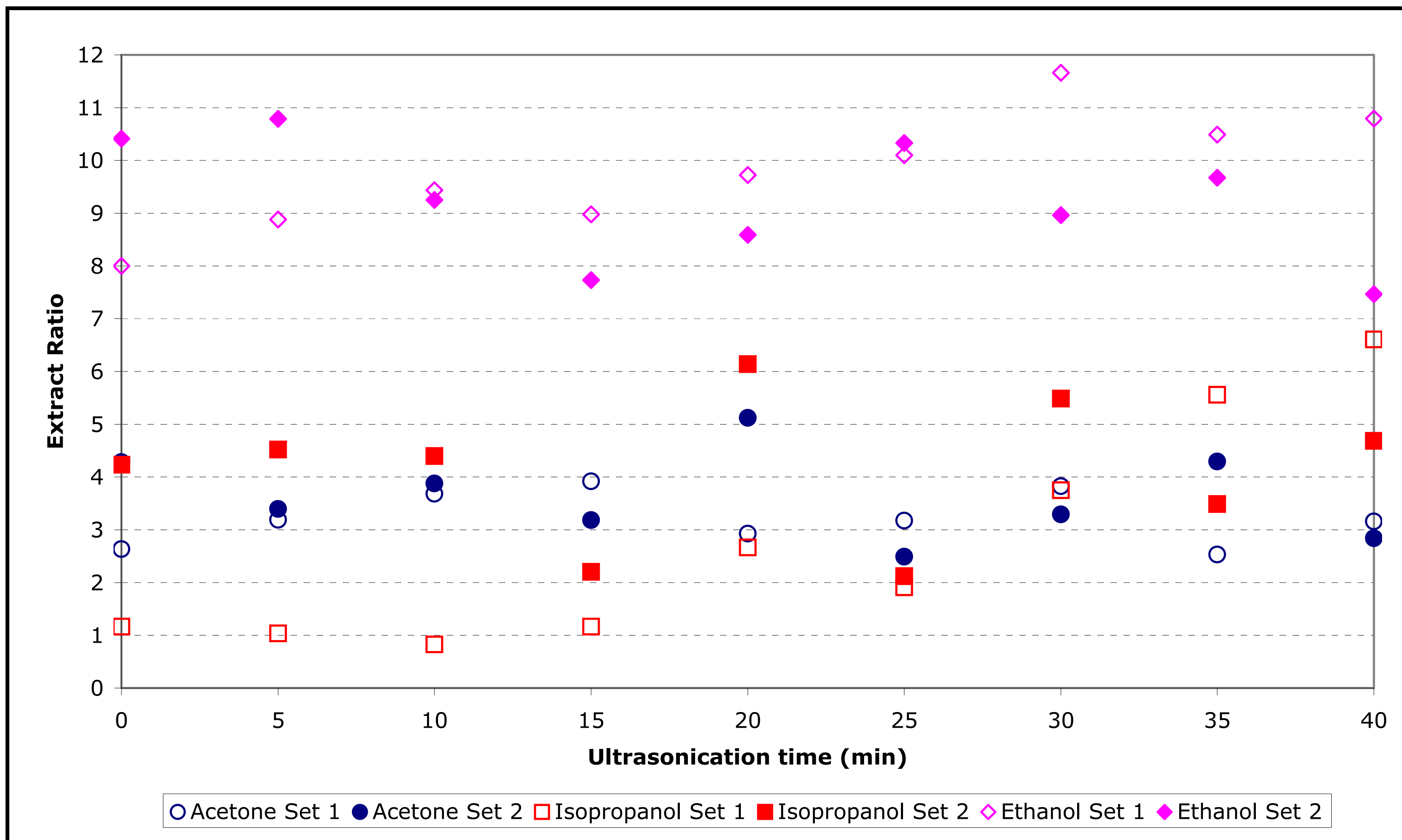

Figure 25 Extract Ratio vs. Ultrasonication time graph for all solvents

B4 ENHANCEMENTS IN THE SEPARATION OF THE SOLID AND THE LIQUID PHASES

Table 30 No sonication experiments

Samples were extracted in 30 ml Ethanol			
Procedure	**Type of Germ**	**Wheat Germ (g)**	**(g Extract / g germ) x 100**
1	Non roasted	1.63	4.1
1	Roasted	1.08	4.0

Table 31 Ultrasonic extraction for 5 minutes

Samples were extracted in 30 ml Ethanol			
Procedure	**Type of Germ**	**Wheat Germ (g)**	**(g Extract / g germ) x 100**
1	Non roasted	1.63	6.9
1	Roasted	1.08	6.1
2	Non roasted	1.85	5.6
2	Roasted	1.80	6.1
3	Non roasted	2.11	4.6
3	Roasted	1.50	7.5
4	Non roasted	1.25	8.8
4	Roasted	1.66	9.0

Table 32 Ultrasonic extraction for 25 minutes

Samples were extracted in 30 ml Ethanol			
Procedure	**Type of Germ**	**Wheat Germ (g)**	**(g Extract / g germ) x 100**
1	Non roasted	1.73	10.2
1	Roasted	1.76	9.6
2	Non roasted	1.47	7.7
2	Roasted	1.46	8.3
3	Non roasted	1.77	6.0
3	Roasted	1.98	7.7
4	Non roasted	2.28	7.5
4	Roasted	1.68	8.9

B5 TOTAL POLYPHENOL CONTENT

B5.1 TOTAL POLYPHENOL CONTENT OF SET 1

Table 33 TPC of nonroasted wheat germs set 1

Each sample weighs 2.00 g wheat germ and extracted with ethanol			
Sample	**Time (min)**	**Absorbance (Nm)**	**mg GA / L solution**
0	0	0.20	93
1	5	0.24	116
2	10	0.27	128
3	15	0.27	129
4	20	0.36	172
5	25	0.37	174
6	30	0.44	208
7	35	0.28	132
8	40	0.39	187

Table 34 TPC of roasted wheat germs set 1

Each sample weighs 2.00 g wheat germ and extracted with ethanol			
Sample	**Time (min)**	**Absorbance (Nm)**	**Mg GA / L solution**
0	0	0.21	98
1	5	0.24	116
2	10	0.33	155
3	15	0.30	143
4	20	0.36	172
5	25	0.19	91
6	30	0.34	164
7	35	0.32	151
8	40	0.36	173

B5.2 TOTAL POLYPHENOL CONTENT OF SET 2

Table 35 TPC of nonroasted wheat germs set 2

Each sample weighs 2.00 g wheat germ and extracted with ethanol			
Sample	**Time (min)**	**Absorbance (Nm)**	**mg GA / L solution**
0	0	0.13	64
1	5	0.13	62
2	10	0.20	96
3	15	0.13	64
4	20	0.22	107
5	25	0.37	175
6	30	0.49	232
7	35	0.40	190
8	40	0.40	191

Table 36 TPC of roasted wheat germs set 2

Each sample weighs 2.00 g wheat germ and extracted with ethanol			
Sample	**Time (min)**	**Absorbance (Nm)**	**mg GA / L solution**
0	0	0.14	67
1	5	0.15	70
2	10	0.34	163
3	15	0.33	158
4	20	0.40	191
5	25	0.25	121
6	30	0.35	167
7	35	0.34	162
8	40	0.42	199

B5.3 TOTAL POLYPHENOL CONTENT OF SET 3

Table 37 TPC of nonroasted wheat germs set 3

Each sample weighs 2.00 g wheat germ and extracted with ethanol			
Sample	**Time (min)**	**Absorbance (Nm)**	**mg GA / L solution**
0	0	0.17	82
1	5	0.33	156
2	10	0.40	191
3	15	0.33	157
4	20	0.35	167
5	25	0.25	118
6	30	0.34	160
7	35	0.31	150
8	40	0.41	197

Table 38 TPC of roasted wheat germs set 3

Each sample weighs 2.00 g wheat germ and extracted with ethanol			
Sample	**Time (min)**	**Absorbance (Nm)**	**mg GA / L solution**
0	0	0.16	77
1	5	0.29	137
2	10	0.36	171
3	15	0.37	175
4	20	0.39	185
5	25	0.29	138
6	30	0.40	190
7	35	0.33	159
8	40	0.41	196

B5.4 AVERAGE TOTAL POLYPHENOL CONTENTS

Table 39 Average TPC of roasted wheat germs

Each sample weighs 2.00 g wheat germ and extracted with ethanol		
Sample	**Time (min)**	**mg GA / L solution**
0	0	81
1	5	108
2	10	163
3	15	159
4	20	183
5	25	116
6	30	173
7	35	157
8	40	189

Table 40 Average TPC of nonroasted wheat germs

Each sample weighs 2.00 g wheat germ and extracted with ethanol		
Sample	**Time (min)**	**mg GA / L solution**
0	0	80
1	5	111
2	10	138
3	15	117
4	20	148
5	25	156
6	30	200
7	35	157
8	40	192

B5.5 STATISTICAL ANALYSIS OF NONROASTED WHEAT GERMS

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.69
R Square	0.48
Adjusted R Square	0.46
Standard Error	34.91
Observations	27

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	28073.83	28073.83	23.03	6.27E-05
Residual	25	30473.49	1218.94		
Total	26	58547.32			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	94.32	12.39	7.61	0.00	68.80	119.83	68.80	119.83
X Variable 1	2.50	0.52	4.80	0.00	1.43	3.57	1.43	3.57

RESIDUAL
OUTPUT

Observation	*Predicted Y*	*Residuals*		Pure Error
1	94.32	-1.27		434.89
2	94.32	-30.46		
3	94.32	-12.22		
4	106.80	9.01		4438.37
5	106.80	-45.04		
6	106.80	48.82		
7	119.29	8.90		4691.12
8	119.29	-23.67		
9	119.29	71.61		
10	131.78	-2.73		4519.41
11	131.78	-67.78		
12	131.78	24.79		
13	144.27	27.49		2632.56
14	144.27	-37.70		
15	144.27	22.49		
16	156.76	17.38		2123.01
17	156.76	18.05		
18	156.76	-38.71		
19	169.25	38.42		2684.98
20	169.25	62.61		
21	169.25	-9.39		
22	181.74	-49.35		1774.98
23	181.74	8.65		
24	181.74	-32.16		
25	194.22	-7.65		59.06
26	194.22	-3.22		
27	194.22	3.16	Sum	23358.37

	df	SS	MS	**F Ratio**
LOF	7	7115.12	1016.45	**0.78**
Pure Error	18	23358.37	1297.69	

From the F test it is found that $F > F_{CRITICAL}$ however

From the lack of fit test it is found that lack of fit is insignificant,

Therefore the **linear regression is significant**.

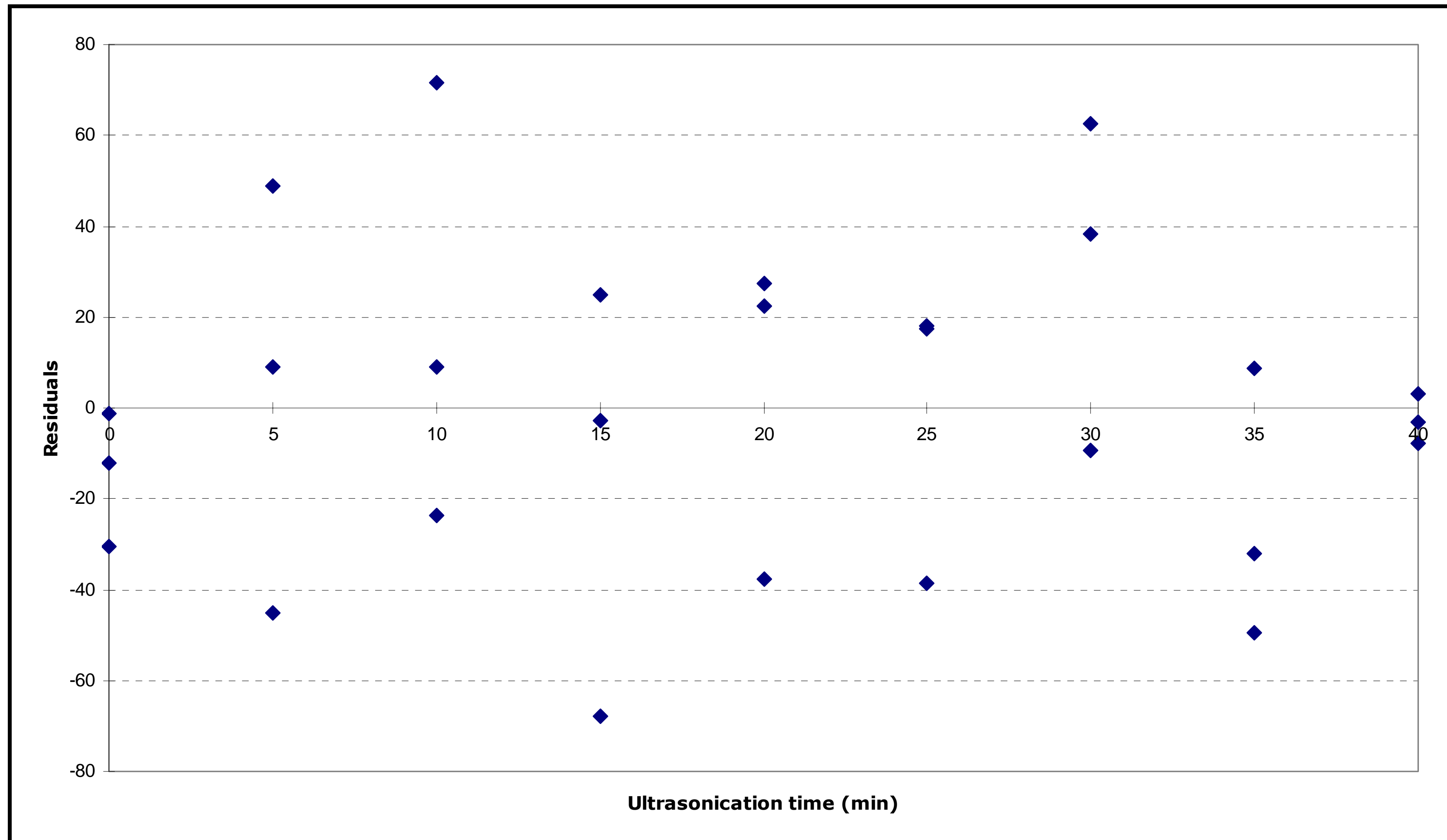

Figure 26 Residual TPC plot of nonroasted wheat germs

B5.6 STATISTICAL ANALYSIS OF ROASTED WHEAT GERMS

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.64
R Square	0.40
Adjusted R Square	0.38
Standard Error	30.49
Observations	27

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	15818.97	15818.97	17.02	3.59E-04
Residual	25	23240.68	929.63		
Total	26	39059.65			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	110.18	10.82	10.18	0.00	87.90	132.46	87.90	132.46
X Variable 1	1.87	0.45	4.13	0.00	0.94	2.81	0.94	2.81

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*		Pure Error
1	110.18	-11.94		519.88
2	110.18	-43.66		
3	110.18	-32.85		
4	119.55	-3.60		2397.64
5	119.55	-49.84		
6	119.55	17.92		
7	128.93	26.07		124.98
8	128.93	33.88		
9	128.93	41.88		
10	138.30	4.89		491.28
11	138.30	19.79		
12	138.30	36.22		
13	147.68	24.42		181.95
14	147.68	43.04		
15	147.68	37.32		
16	157.05	-66.24		1142.54
17	157.05	-36.53		
18	157.05	-18.96		
19	166.43	-2.76		399.98
20	166.43	0.48		
21	166.43	23.19		
22	175.80	-24.32		57.37
23	175.80	-13.94		
24	175.80	-16.85		
25	185.18	-11.99		396.07
26	185.18	14.01		
27	185.18	10.35	Sum	5711.69

	df	SS	MS	**F Ratio**
LOF	7.00	17528.99	2504.14	**7.89**
Pure Error	18.00	5711.69	317.32	

From the F test it is found that $F > F_{CRITICAL}$ but,

From the lack of fit test it is found that lack of fit is significant,

So it is concluded the **linear regression is insignificant**.

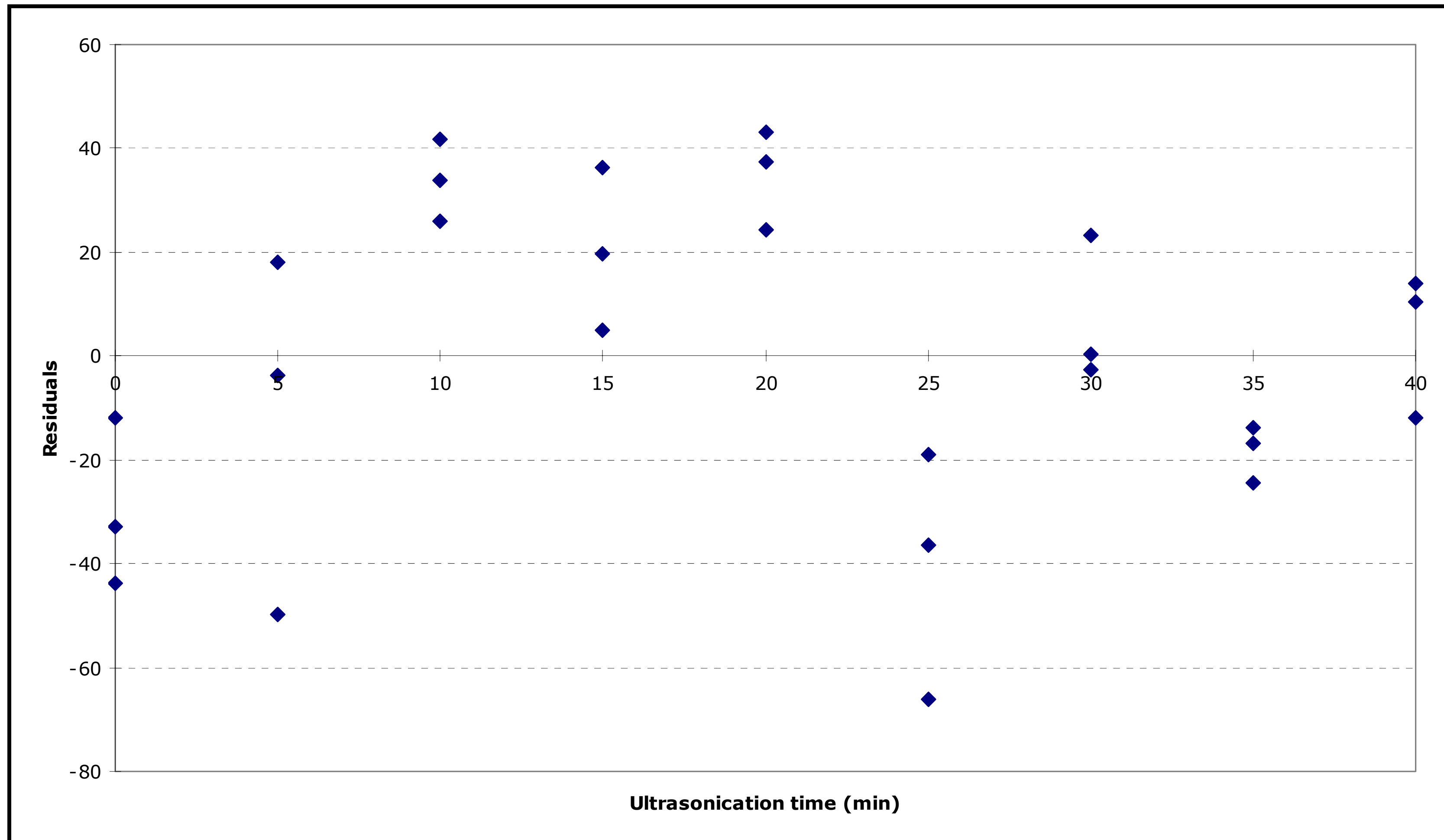

Figure 27 Residual TPC plot of roasted wheat germs

APPENDIX C

WAVELENGTH CHECK

The wavelength is checked using the UV Spectrophotometer, Hitachi U – 3200, which is shown in Figure 28. For the range 700 – 800 nm the wavelength check graph is given in Figure 29.

Figure 28 UV spectrophotometer, Hitachi U –3200

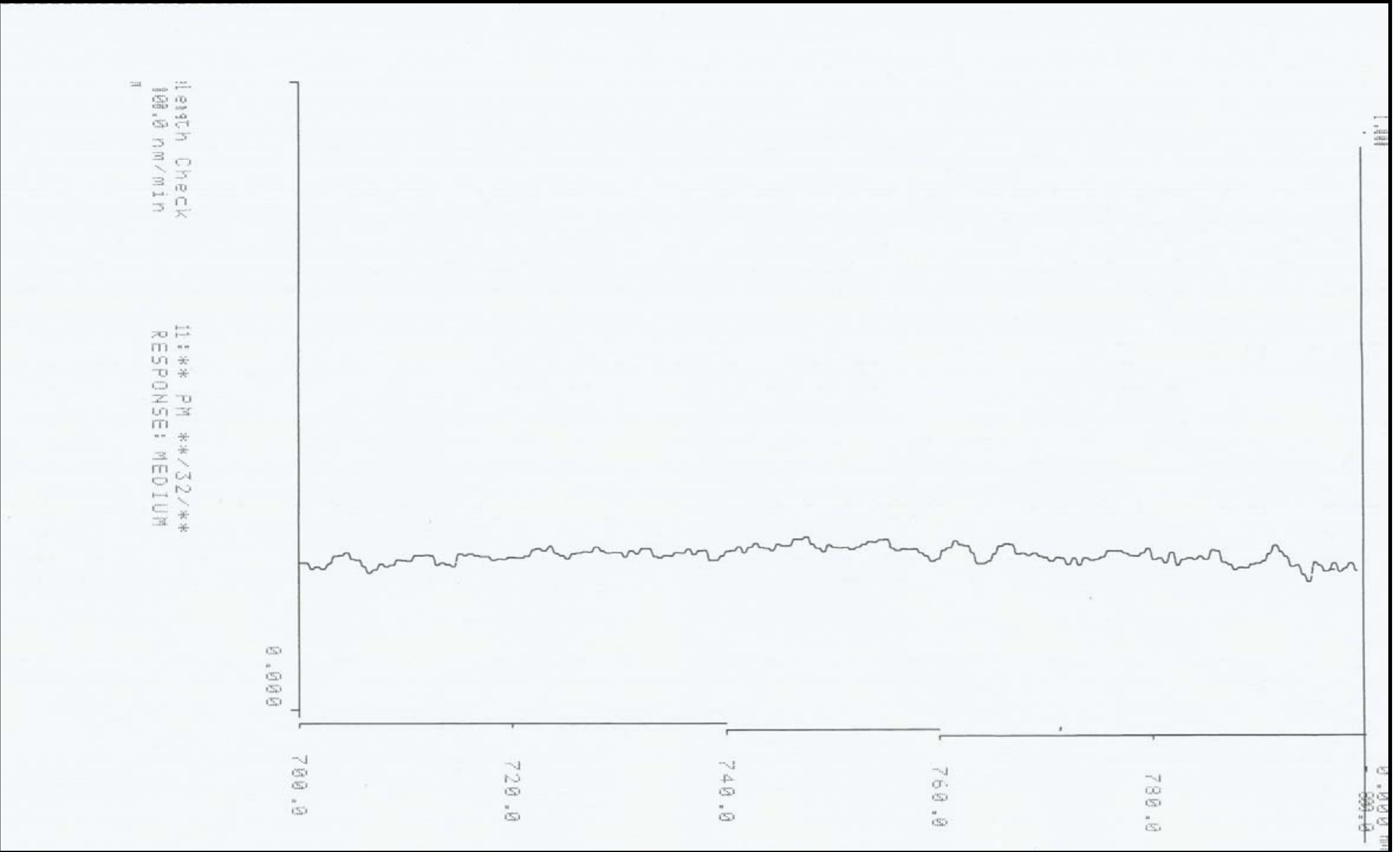

Figure 29 Wavelength check between 700 – 800 nm

APPENDIX D

COMPOSITION OF THE FOLIN – CIOCALTEU'S REAGENT

Table 41 Composition of the Folin-Ciocalteu's Phenol Reagent (Sigma)

SIGMA-ALDRICH

Material Safety Data Sheet

Date Printed: 18/JAN/2005
Date Updated: 15/MAR/2004
Version 1.3
According to 91/155/EEC

1 - Product and Company Information

Product Name	FOLIN & CIOCALTEU'S PHENOL REAGENT
Product Number	F9252
Company	Sigma-Aldrich Chemie GmbH Riedstrasse 2 89555 Steinheim
Technical Phone #	49-89-6513(0)-1444
Fax	49-7329-97-2319
Emergency Phone #	49 7329 97 2323

2 - Composition/Information on Ingredients

Product Name	CAS #	EC no	Annex I Index Number
FOLIN-CIOCALTEU'S PHENOL REAGENT	None	None	None

Ingredient Name	Percent	CAS #	EC no	Annex I Index Number
WATER	57.5	7732-18-5	231-791-2	None
LITHIUM SULFATE Symbols: Xn R-Phrases: 22 Harmful if swallowed.	15	10377-48-7	233-820-4	None
SODIUM TUNGSTATE DIHYDRATE Symbols: Xn R-Phrases: 22 Harmful if swallowed.	10	10213-10-2	236-743-4	None
HYDROCHLORIC ACID >=25% Symbols: C R-Phrases: 34 37 Causes burns. Irritating to respiratory system.	10	7647-01-0	231-595-7	017-002-01-X
PHOSPHORIC ACID, 85 WT% SOLUTION IN WATER Symbols: C R-Phrases: 34 Causes burns.	5	None	None	None
MOLYBDIC ACID SODIUM DIHYDRATE	2.5	10102-40-6	231-551-7	None