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SCHOOL OF CHEMICAL AND BIOENGINEERING

Optimization and characterization of antioxidant activity from green tea (*Camellia sinensis*) and evaluation of its preservative effect

A Thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Science in Chemical Engineering (Food Engineering Stream)

BY

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This is to certify that the thesis prepared by Shimelis Shumi, entitled: **Optimization and characterization of antioxidant activity from green tea (*Camellia sinensis*) and evaluation of its preservative effect** and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Chemical Engineering (Food Engineering Stream) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

The study was conducted with the aim of optimizing and characterizing antioxidant activity of Camellia sinensis and evaluating its preservative effect on Niger seed and Soybean oils. Camellia was prepared by investigating the effect of distilled water concentrations of 100, 150, and 200ml, extraction temperature 80, 85 and 90 ℃ and contact time of 10, 35 and 60 min on its antioxidant activity and extract yield. Based on ANOVA analysis, extraction parameters have significant positive effect ($P<0.05$) on Camellia antioxidant activity and its extract yield. The best levels of extraction parameters for higher antioxidant activity and extract yield were distilled water concentration of 200ml for 60min at 90 ℃. As DPPH free radical scavenging activity results shown the highest antioxidant activity of Camellia was 96.1%. On the other hand extraction parameters (solvent concentrations, extraction temperature & contact time) were optimized. The best extraction conditions were 200ml, 88 ℃ and 35 min and the results were yield (79.850%) and Antioxidant activity (96.9810%). The preservative effect of Camellia was also studied by performing Free Fatty Acids, Peroxide Value/Acid value on Niger seed and Soybean oils. In the study, all samples were treated with 0, 1 and 2ml levels of Camellia extract and analysis were conducted on weekly basis. Compared to Camellia extract containing Niger seed and Soybean oils, control sample showed higher Free Fatty Acid and Peroxide Values in each storage weeks. Samples treated with 2ml Camellia extract were shown lower Free Fatty Acid and Peroxide Values in each storage weeks. Based on the results, samples with 2ml Camellia extract significantly improved the oxidative stability of Niger seed and Soybean oils. Hence, Camellia has antioxidant activity and preservative effect as evaluated on Niger seed and Soybean oils.

Keywords: *Antioxidant activity, green tea extract, Camellia sinensis, response surface methodology, DPPH free radical scavenging activity, preservative effect.*

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I dedicate this work to my sweet kid Natan Shimelis; my kid may GOD Bless you!.

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List of Acronyms

ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemistry
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxyl toluene
DPPH	1, 1 Diphenyl-1-picrylhydrazyl
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
FAO	Food and Agricultural Organization
FCCCD	Face Centered Central Composite Design
FFA	Free Fatty Acid
GTPs	Green tea polyphenols
IT	Induction time
PV	Peroxide Value

Appendices

CHAPTER ONE

INTRODUCTION

1.1. Background

Most food products are perishable and need protection from spoilage and deterioration during their preparation, storage, and distribution to give the desired shelf life. Due to the fact that food products are sold mostly far from the production site and thus extension of safety and storage is mandatory. The major cause of deterioration in fat containing food products is rancidity. Rancidity brings about a significant change in product odour, taste, colour, texture, and nutritive value. Progressing oxidation results in complete spoilage of foods. Use of antioxidants can reduce problems caused by rancidity, thus are used frequently to retard oxidation processes in the food industry (Allen and Hamilton, 1994).

Antioxidants are important ingredients in food processing sectors. As their name implies, their role is to inhibit the development of oxidative rancidity in fat containing foods, particularly meat, dairy products, and fried foods. It was reported that antioxidants are 'substances that when present in low concentrations with those of an oxidisable substrate, significantly retard oxidation of that substrate.'(Halliwell and Guteridge, 2007)

It is well known that plants are the richest sources of bioactive phytochemicals and antioxidant nutrients (Ellessa *et al.,* 2000). It is now broadly accepted that certain classes of plant –based compounds such as dietary fiber, phenolic acids, flavonoids, vitamins, and antimicrobial agents and neuropharmacological agents play preventive role against the incidence of some common disease like cancer, cardiovascular and neurodegenerative disorders (Siddhuraju and Backer,2007;Fan *et al*.,2007;Liu *et al*.,2008). In the modern food science era, the foods, which in the addition to imparting normal nutritive value also have disease protecting and physiological benefits, are known as" functional foods." The term, physiological functional foods, first appeared in Nature News in 1993 with the title "Japan explores the boundaries between food and medicine.

In recent years there is a much focus on replacing synthetic food additives which might have adverse effect with those of plant-based natural ones (Paradiso *et al*.,2008, Descalzo and Sancho; 2008). The increasing uses of herbal products demand extra attention with particular focus on their safety, effectiveness, and drug interactions. Over the last few decades, a substantial body of scientific evidence is available demonstrating wide range of pharmacological and nutraceutical activities of medicinal herbs (Burt, 2004; Celiktas *et al*., 2007; Edris, 2007). These include antioxidant, antimicrobial, and anticancer, anti-inflammatory activities.

The essential oils and herbs derived extracts are gaining much recognition as a potential source of natural and safer antioxidants and bioactive (Burt *et al*., 2003; Burt, 2004). Essential oils of some spices and herbs such as sage, oregano, thyme, tea and satureja etc. have shown their antioxidant potential (Ruberto and Baratta, 2000; Rota *et al*., 2008) and thus can be used as natural antioxidants for the protection of fats/oils and related products (Burt, 2004; Scchetti *et al*., 2005; Bozin *et al*., 2006).

Many species of spices and herbs food materials are active (Kasuga *et al.,* 1988; Hirosue et al., 1988) mainly because of their content of phenolic compounds. The most important representatives of this group of substances are leaves from tea bush, Camellia sinensis or C. assamica L., (Balentine and Paetau-Robinson, 1996). Green, oolong, and black teas are produced; depending on the technology of leaves processing (Xie *et al*., 1993). Green tea contains a high percentage (about 20%) of catechins and related compounds (Chen *et al*., 1996). The mixture mainly consists of catechins, epicatechins, galloctechins, and the respective gallates. Extracts from green tea are, therefore, very active; their activity is comparable to that of the synthetic antioxidants.

The antioxidant activities of herbs and spices extracted with solvents, such as ethanol, methanol, or acetone, have been evaluated in various test system. So far, many investigations on the antioxidant activities of ethanol or methanol extracts of tea, rosemary, sage, etc. have been carried out in hydrophilic and lipophilic test systems. Spices and herbs may fulfill more than one function to foods to which they are added (Shelef *et al*, 1980). In addition to imparting flavor, certain spices prolong the storage life of foods by a bacteriostatic or bactericidal activity and some prevent rancidity by antioxidant activity. Tea (*Camellia sinensis*) and its phenolic constituents are reported to have antioxidant activity in vitro that minimizes the oxidizability of

fatty acids and α-tocopherol (Luo *et al.*, *1997*) and has potential for use in several food products (Madhavi *et al.*, 1996).

It is of a great interest to consumers and nutritionists to quantify the antioxidant of various foods because antioxidants have a great important to human beings. Antioxidant functions can be defined as the ability of a compound to reduce pro-oxidant agents (Prior and Cao, 1999). Many methods have been proposed for measuring the antioxidant activity (Gordon, 2001; Wan, 1994). The oldest and the most precise method is the Schaal oven test, where the sample is stored at 40°C, 50°C, or 60°C in the dark under free access of oxygen. Changes are monitored either by determining the peroxide value or increase of weight (García-Mesa *et al*., 1993). The Rancimat or Oil Stability Index (OSI) method is based on the same principle, but the procedure is automatic (Reynhout, 1991).

Numerous studies have been conducted on the antibacterial and antioxidant activity of herbs, spices, and vegetables extract and subsequent effect on the shelf life of different food products. Although, teas which are theaceae family herbs have potential to grow in different parts of our country the antioxidant potential of green, oolong and black teas derived from *Camellia sinensis* leaves are not yet well investigated in relation to the shelf-life of fat containing food products. Therefore, the present study is aimed to extract and characterize antioxidant from green tea and evaluate its preservative effect on Niger seed oil and soybean oil.

1.2. Statement of the problem

Fats and oils are essentials ingredients in the formulation and processing of foods in the food industry. The quality of lipid containing products is determined based on the oxidative stability of fats and oils. Autoxidation of fats or oils is a complex process initiated by free radical reactions involving unsaturated fatty acids. Hence, fats and oils containing unsaturated fatty acids are highly susceptible to oxidation. Thus, development of food preservation processes has been driven by the need to keep the oxidative stability of fats and oils and extend the induction period or shelf-life of foods. The oxidative deterioration of fats and oils are a great concern in the shelf life of lipid- containing foods, due to decrease food safety and nutritional quality by forming toxic products and secondary reaction products during cooking or processing. As far as fats and oils are concerned, the problems associated with lipid oxidation can be countered by the application of antioxidants.

Synthetic antioxidants have been used to retard or minimize oxidative deterioration of foods, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ). However, recently, consumers have rejected synthetic antioxidants because of their carcinogenicity.

The increasing preference for natural foods has obliged the food industries to include natural antioxidants in various products to delay oxidative degradation of lipids, improve quality and nutritional value of foods, and replace synthetic antioxidants. Therefore, investigation of natural antioxidants has been a major research interest in screening spices, herbs and plant materials for possible antioxidant potential.

Thus, this research has aimed to solve problems associated with rancidity by the application of natural antioxidant extracted, optimized and characterized from green tea and its preservative effect on soybean and Niger seed oils.

1.3. Objectives

1.3.1. General Objective

The general objective of the thesis was to optimize and characterize antioxidant from green tea and evaluate its preservative effect on Niger seed and soybean oils.

1.3.2. Specific Objectives

The specific objectives of this study were to:

- study the effect of extraction parameters on Camellia antioxidant activity and extract its yield
- evaluate the total antioxidant activity of C.sinensis on Niger seed and Soybean oils
- investigate the effect of Camellia extract concentrations on its antioxidant property
- compare the significance difference antioxidant activity of Camellia and ascorbic acid
- find the Optimum extraction parameters for antioxidant activity and extract yield
- evaluate the validity of experimental model for antioxidant activity and extract yield
- study the preservative effect of Camellia extract on Niger seed oil and soybean oil

1.4. Significance of the study

The typical significances of the research are:

- Oxidation prevention of fat and oil food items
- Preservatives/ additives development
- Eye breakers for farther study

CHAPTER TWO

LITERATURE REVIEW

2.1. Tea (Camellia sinensis)

Tea (*Camellia sinensis)* is the species of plant whose leaves and leaf buds are used to produce Chinese tea. It is of the genus Camellia, a genus of flowering plants in the family Theaceae. The scientific classification of tea is

Kingdom ….……..plantae

Order…….....Ericales

Family…….Theaceae

Genus …….*Camellia*

Species…….C. *sinensis*

Binomial name…….*Camellia sinensis* (L.) Kuntze

Tea (Camellia sinensis) is native to the southern regions of China and parts of India, Laos, Thailand, Vietnam, and Myanmar (Balentine *et al.,* 1998). Tea is said to have first been discovered as a drink and medicine in China around 2737 BC. It was then introduced to Japan during the early 13th century and to Europe in the 16th century, then to America, Africa and other regions of the world (Balentine *et al*., 1998; Chow *et al*., 1990; Wheeler *et al*., 2004). Tea is presently cultivated in over 30 countries around the world and the tea beverage is second only to water in terms of worldwide consumption (Graham et al., 1992). All kinds of tea originate from *Camellia sinensis* (earlier called *Thea sinensis)* which has the two subspecies: *Var sinensis* (China tea) is grown extensively and used in China and Japan and the other subspecies is *Var assamica* (Assam tea) which predominates in South and South-East Asia.

Figure 2.1. Tea Leaves

Tea extracts are powerful antioxidants due to the presence of chemical compounds such as epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin (EC) as shown in Figure 2.1 (Aghamaamadi *et al*., 2011).

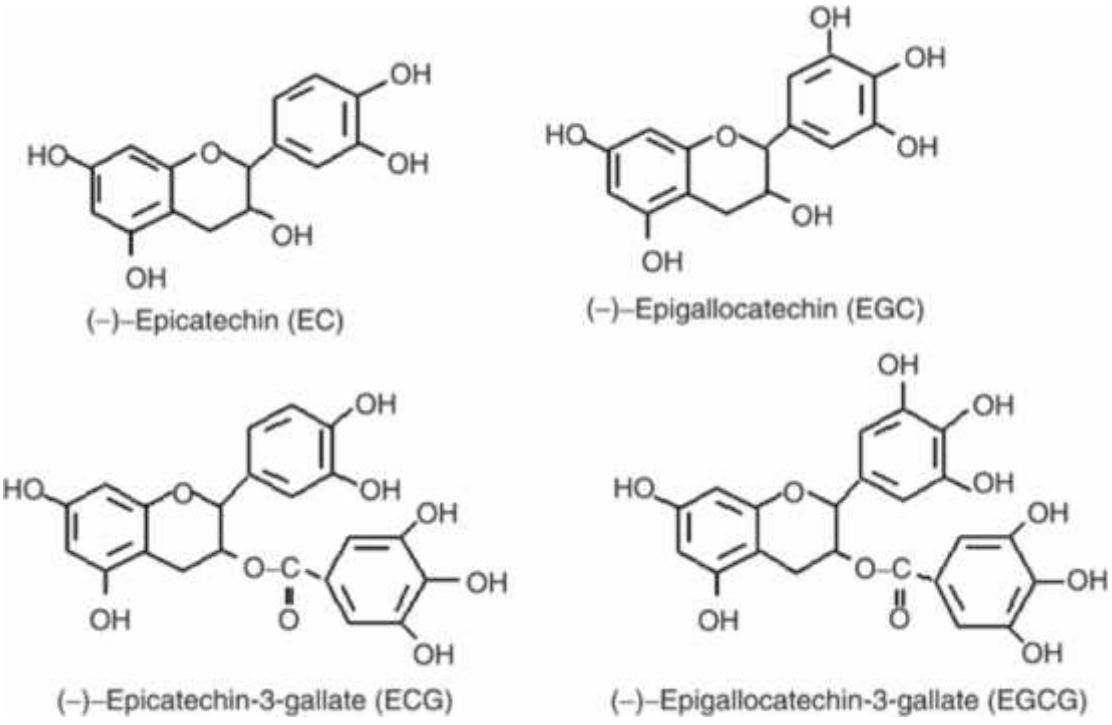


Figure 2.2. Some important tea catechins

2.2. Tea plantation and related aspects

Like other plants need certain requirements to grow, develop and give desired products, tea plantation also requires favorable conditions to give the expected products, such as soil, rainfall, water, nutrients and others. According to (Mauskar, 2007) the following are key factors for ta plantation.

Soil: Tea is grown in a wide range of soil types found in tropical, subtropical and temperate climate conditions. These soil types have developed from diverse parent rock material and under different climate conditions. In China, Indonesia, Sri Lanka, South India, Turkey and Georgia (USSR) tea is mostly grown on sedimentary soils derive from genesis or granite. In north-east India, except in Darjeeling, tea is grown on flat alluvial lands which occupy the vast area of the Brahmaputra Valley in Assam. Peat (bheels) soils that have been drained are successfully used in Cachar to grow tea. But in Kyoto and Kanaya, the main areas of Japanese tea, the crop is grown on soil types derived from volcanic ash and in Taiwan; tea occupies a tract of tertiary rocks derived from a residual formation. However, all these soils have one common characteristic i.e. they grows best in acid soils.

Despite the diversity of soil types on which tea is grown, all the soils exist in high rainfall conditions, as this is the most important climate factor for successful tea growing. These soils get formed under special type of weather (permanent moist conditions) combined with intensive leaching of the products of weathering. The degree of leaching and hence the character of the resulting soil depends not only on the rainfall but also on the temperature. Because of differences in temperature, soils formed in tropical climates are likely to have certain characters different from those formed in the sub-tropical or semi-tropical conditions. The tea areas in tropical climates experience minimal temperature variations as compared with those in sub-tropical climates.

Rainfall: The relationship between tea production and rainfall is well known. Tea grows well in areas having a precipitation of about 1,150 to 8,000 mm. The effect of rainfall is perhaps more manifested by its influence on moisture status of the soil and in inducing vegetative growth. Therefore, distribution of rainfall is as important as the total annual rainfall. It is difficult to say what exactly is water requirement of tea in different phases of its growth and development. It is natural to expect that water requirement of tea would vary according to the prevailing environmental conditions, but it is reasonably assumed that tea on an average may transpire 900

mm per annum. Ideally, the crop water requirement should be such that it does not cause any stress in the plant system.

Humidity: Humidity is of importance in tea physiology primarily because of its influence in determining the loss of moisture by evapo-transpiration. High humidity reduces water loss, but low humidity increases it, and also increases water stress during rainless drought period. In relation to temperature, low humidity may be advantageous because by increasing transpiration rate it also reduces leaf temperature, though the effect will be more pronounced in unshaded conditions. In north-east India the humidity level generally remains high during the harvesting period and this is generally considered to be conducive to growth.

Air Temperature: Tea is grown in tropical to temperate conditions, under a regime of air temperature that varies between –8°C and 35°C; the suitable temperature for growth being the one common to the habitat of the plant, that is, where it grows. But photosynthetic rate of tea is at maximum between 30°C and 35°C, falls rapidly at 37°C, and between 39°C and 42°C there is virtually no net photosynthesis. There is also no uptake of carbon dioxide at about 42°C; respiration may continue up to about 48°C but the leaf is irrevocably damaged. Therefore, there has to be an optimum range of temperature for growth and productivity of tea.

Fertilizer: Fertilization is an important part of the normal intensive production of tea. Tea is normally grown as a long-term monoculture. Without applied fertilizer the supply of nutrients available in the soil will become exhausted leading to mineral deficiencies in the plants, severe reduction in yield and ultimately, to the death of plants and a degraded plantation.

Nutrients are removed from a field of tea in a number of ways. There is an irreversible loss in the crop. The net loss of nutrients in old leaves and pruning depend on the extent to which these items are retained in the field. Soil erosion, drainage of excess water containing nutrients in solution and decomposition to gases create further losses. Uncontrolled weeds also absorb substantial quantities of nutrients.

In addition to oxygen, hydrogen and carbon which are obtainable in air & water, tea plant requires fourteen (14) mineral nutrients for its ideal growth. These are Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), Magnesium (Mg), Sulphur (S), Manganese (Mn), Zinc (Zn), Copper (Cu), Aluminum (Al), Iron (Fe), Boron (B), Molybdenum (Mo) and Chlorine (Cl). Of these essential nutrients mentioned above, the first nine are considered principal nutrients as they are required in relatively larger amounts.

2.2.1. **Major tea plantation countries**

Tea was discovered around 5,000 years ago in China completely by accident. Tea plants are native to South and East Asia and the drink was introduced to Europe during the 16th century via Portuguese colonial rule. Today China is the biggest producer of tea in the world, producing 1.5 million tons last year. After China, India, Kenya, Sri Lanka, Turkey, Vietnam, Iran, Indonesia, Argentina and Japan are the largest producers. The following table 2. Shows international scenario of tea plantation or cultivation of major countries in hectares, World Trade Report from and Ethiopian Investment Agency 2007-2012 G.C.

Table 2.1. Area under tea in different countries (area in hectares).

Country	2007	2008	2009	2010	2011	2012
China	1023933	1061864	1084200	1134600	1115300	1103000
India	407647	416269	420289	425966	427065	431245
Sri Lanka	222905	221758	221836	NA	NA	NA
Indonesia	124093	134934	138736	128503	NA	514185
Kenya	84400	97020	101845	110222	112556	117457
Turkey	83470	90575	89345	76971	76609	76743
Japan	60200	58500	56700	54500	53700	52700
Vietnam	58100	59900	60000	64000	71000	64000
Argentina	42350	41276	41406	NA	40000	39000
Uganda	20905	20905	20905	20905	20905	20905
Tanzania	18875	18875	19415	19881	20153	20564
Malawi	18790	18204	18587	18801	18963	18986
Ethiopia	1800	NA	NA	2000	2400	2700

NA: Not Available

2.2.2. Tea plantation in Ethiopia

In Ethiopia, tea is mostly grown in the highland dense forest regions where the land is fertile and thus the use of fertilizer is very minimal. Climatic conditions, the type of soil and the method of processing combine to produce quality Ethiopian teas. Manual weeding is employed thanks to the availability of abundant and cheap labor. Ethiopian tea is highly sought after thanks to its mostly organic cultivation. The "International Gold Star" award for quality was recently given by B.D.I. in Madrid, Spain, to Tea Production and Marketing Enterprise one of the major Ethiopian tea exporters.

So far the largest commercial Tea Plantations in Ethiopia are those of Wush Wush and Gumaro located in South Western Ethiopia. These formerly state-owned Plantations together with the Tea Processing and Packing Factory in Addis Ababa were bought by Ethio Agri-CEFT in 2000.

The following table shows certain areas of Ethiopia where tea plantation is found, Wush Wush and Gumaro tea plantations.

Table2.2. Regions of tea plantations in Ethiopia.

Brand name	Location	Distance from Addis Ababa (km)	Altitude (m)	Annual Rainfall (mm	Temp. (°C)	Soil property
Wush Wush Tea plantation	Kaffa Zone	460 SW of Addis	1900	1820	10-30	fertile & rich in org. matter
Gumaro Tea Plantation	Illubabor (Oro. Reg.)	637 SW of Addis	1718	2089	10-30	fertile & rich in org. matter

Source: Ethio Agri- CEFT.

Currently the total annual production of the two plantations is about 5700 tons. But, through improved management and processing, both productivity and quality are increasing. As a result, the annual total production is expected to reach about 7000 tons.

2.3. Production and Processing of tea

In the fields, plucking is done on a ten day basis from 0-1,200m and every three weeks from 1,200m to over 2,000m. Tea is either 'fine plucked', only the flush (two leaves and the bud) or 'coarse plucked' (a sprig with more than two leaves). Weighing is done after each tea plucker

has picked between 20-30 kg of leaves, depositing them in wicker baskets or gunny sacks which are transported to the factory.

There are two methods of tea processing can be roughly distinguished, namely the **orthodox method** and **CTC-production method**. The orthodox method is more comprehensive and time consuming compared with CTC production. In general, whatever the method of process is, based on the oxidation of the polyphenols in the tea leaves during the fermentation process, tea has been classified into three types: **green tea, black tea, and oolong tea** (Graham et al., 1992).

Green tea: The intent during the production of green tea is to preserve the healthy, natural and active substances of the fresh leaves so they may be released into the cup at the time of infusion. After picking, the green leaves are spread out in the hot air to wither. Once they have become soft and pliable, they are traditionally pan-fried in woks. This prevents the leaves from oxidizing (usually called fermenting) as it occurs during the production of black tea. The subsequent rolling gives the leaves their style: twisted, curly or balled as well as increased durability. Rolling also helps to regulate the release of the natural substances and flavor during the steeping. In the final step, the leaves are dried by firing whereby the natural fragrances and flavors are stabilized; the leaves keep their green color.
The resulting green teas are high in nutrients and minerals; their health benefits are the subject of a great number of medical studies.

Oolong tea: Almost exclusively produced in China and Formosa (Taiwan), oolong teas fall between the unfermented green teas and the fully fermented black teas. They are processed to be full-bodied teas and are therefore made from larger, more mature leaves.

Immediately upon plucking, the leaves are spread out in direct sunlight to wither. Withering reduces the moisture content and softens the leaves. The leaves are then put into bamboo baskets and shaken briskly to bruise the leaf edges. In the next step, the leaves are spread out in the shade to dry. The process of shaking and spreading of the leaves is repeated numerous times. The bruised leaf edges begin to turn red through the oxidation process (fermentation) while the centers of the leaves remain green.
The amount of fermentation depends on the type of oolong and can vary from approximately 20% for a "green" oolong, to 60 % for a classic Formosa oolong. Once the desired level of

fermentation is reached, the process must be stopped immediately. This is done by pan-firing the leaves at high temperatures, which produces lower moisture content than is found in green tea, and ensures a longer shelf life for oolongs.

Black tea: Unlike green or oolong teas, black teas, during the production process, undergo a full oxidation (usually called fermentation) which causes the leaves to turn black and gives them their characteristic flavor. After picking, the green leaves are spread out on tiers of racks to wither for about 12 to 18 hours. During the long withering process, the leaves lose most of their moisture, becoming soft and pliable so they can be rolled.

During the rolling, the membranes of the leaves are broken, allowing the juices and essential oils that give the tea its aroma to develop. After rolling, the leaves are brought into large, cool, humid rooms where they are spread in layers of about four inches high to oxidize. During the oxidation process, the leaf color darkens, and the initially bitter juices mellow. The characteristic flavors of black tea ranging from flowery to fruity, nutty and spicy – begin to emerge. The oxidation process must be stopped at the point where the aroma and flavor have fully developed. This is done by firing the leaves in large ovens. The flavorful juices dry on the surface of the leaves and remain relatively stable until exposed to boiling water during infusion.

In the last step, the leaves must be sorted by size. During the production process, many tea leaves are broken or crushed so that the finished tea consists of full leaves, broken leaves and smaller particles (fanning). Since the necessary steeping time increases with the size of the leaf, the tea must be sorted into lots of equal leaf size. The following flow sheet shows the production process of the two commonly processed tea types.

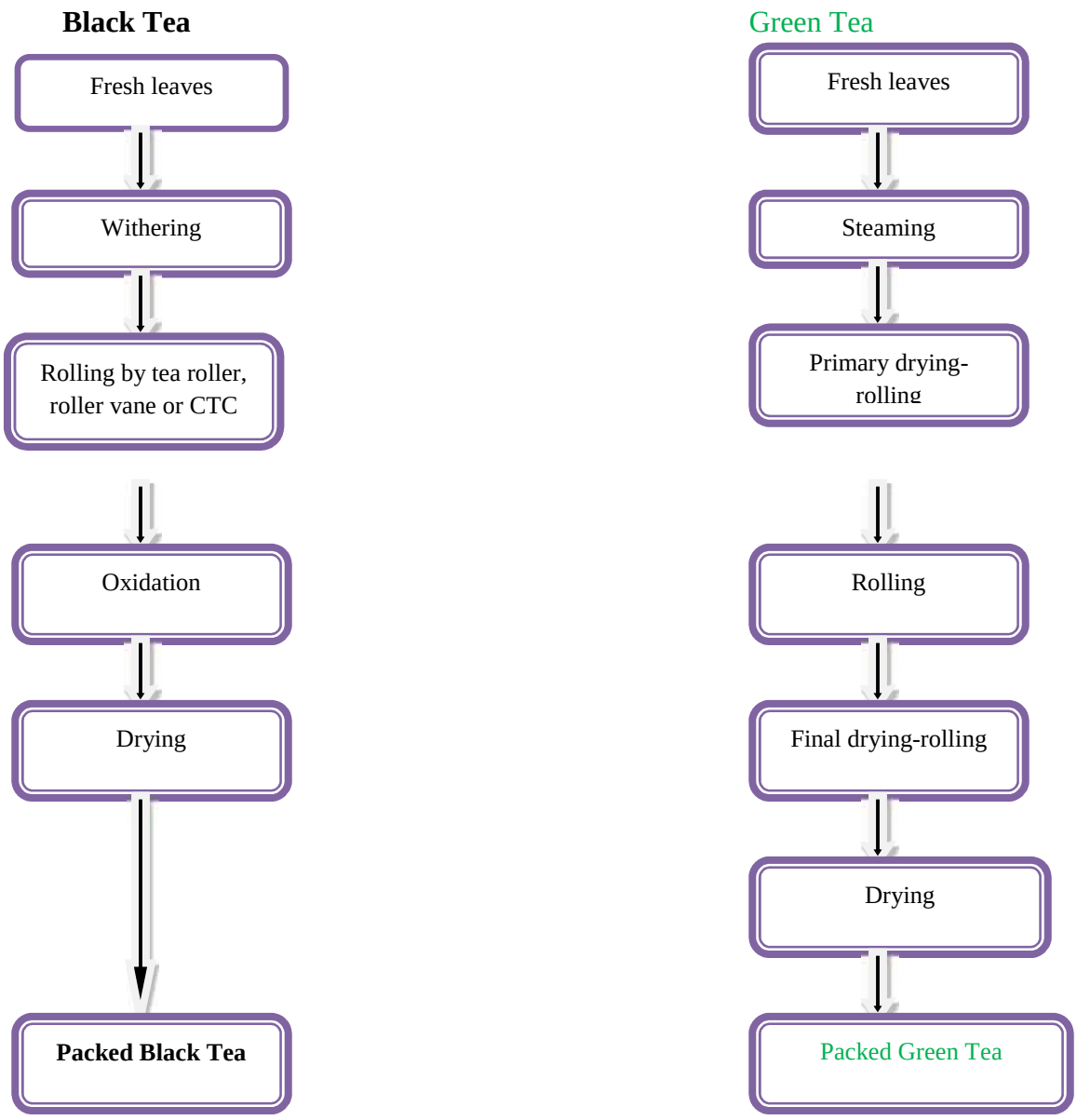


Figure 2.3. Green and Black Tea Production Process Flow Sheet

2.4. Chemical composition of green tea

The chemical composition of green tea is complex. Green fresh tea leaves contain, on average, 3-4% of alkaloids known as methylxanthies, such as caffeine, theobromine, and theophylline. In addition, there are phenolic acids such as gallic acids and characteristic amino acid such as theanine present (Taheri *et al*., 2011)

Green tea contains polyphenols, which include flavanols, flavandiols, flavonoids, and phenolic acids; these compounds may account for up to 30% of the dry weight. Most of the green tea polyphenols (GTPs) are fla vonols, commonly known as catechins. Products derived from green tea are mainly extracts of green tea in liquid or powder form that vary in the proportion of polyphenols (45-90%) and caffeine content (0.4-10%). The major flavonoids of green tea are various catechins, which are found in greater amounts in green tea than in black or Oolong tea (Taheri *et al*., 2011). There are four kinds of catechins mainly find in green tea: epicatechin, epigallocatechin, epicatechin-3-gallate, and EGCG (Taheri *et al*., 2011). The preparation methods influence the catechins both quantitatively and qualitatively; the amount of catechins also varies in the original tea leaves due to differences in variety, origin, and growing conditions (Aghamaamadi *et al*., 2011). The preparation of fresh green tea cannot totally extract catechins from the leaves; therefore, the concentration found differs from the absolute values determined through the complete extraction of leaves (Aghamaamadi *et al*., 2011). Moreover, catechins are relatively unstable and could be quantitatively and qualitatively modified during the time frame of an experiment (Aghamaamadi *et al*., 2011). Thus, comparison of ingested doses in animal studies is not possible because the catechins quantification before administration is often not known. The following table summarizes the chemical composition of green tea.

Table 2.3. Chemical composition of Green tea (Ikuo *et al*, 2010).

Compounds	Contents (%)
Protein	15
Amino acids	4
Fiber	26
Others carbohydrate	7
Lipids	7
Pigments	2
Minerals	5
Phenolic compounds	30
Oxidized phenolic compounds	0

On the other hand, Table 2.4 below shows phenolic compounds and its molecular weight of green tea.

Table 2.4. Phenolic compounds (Flavanols) in tea flush (Angshuman, 2012).

Particulars	short	Formula	Molecular wt.	Dry wt. (%)
Flavanols				
(-)-Epicatechin	(-)-EC	$C_{15}H_{14}O_6$	290	1-2
(-)-Epicatechin gallate	(-)-ECG	$C_{18}H_{18}O_{10}$	442	3-6
(-)-Epigallocatechin	(-)-EGC)	$C_{15}H_{14}O_7$	306	3-6
(-)-Epigallocatechin gallate	(-)-EGCG	$C_{22}H_{18}O_{11}$	458	9-12
(+)- Catechin	(+)-C	$C_{15}H_{14}O_6$	290	1-2
(+)-Gallocatechin	(+)-GC	$C_{15}H_{14}O_7$	290	1-2
(+)-Gallocatechin gallate	(+)-GCG	$C_{22}H_{18}O_{11}$	458	-
(+)-Catechin gallate	(+)-CG)	$C_{18}H_{18}O_{10}$	442	-
Total polyphenols				25-35

2.5. Antioxidant properties of green tea leaf

Green tea extract has strong antioxidant due to the presence of (+)catechin, (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epigallocatechin-3-gallate (EGCG). Catechin is a compound which does not evaporate and it contained about 8-15% from the dry weight of plant (Mohd, 2013).

Young leaves are discovered to have higher antioxidant activity than mature leaves. From previous studies, green tea was found to contain higher antioxidant activity than black tea. Total phenolic content, DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity, ferric reducing antioxidant power (FRAP) and ABTS [2,2'-azinobis-(3-ethylbenzothiazoneline-6-sulfonic acid)] decolorization assays conducted showed that green tea has higher antioxidant properties than black tea (Gadow *et al*., 1997; Chan *et al*., 2007; Pilar *et al*., 2008), .

In the food industry, the catechins have proven useful because of their high potency for preventing lipid peroxidation of oil-containing foods; they scavenge free radicals and stop the auto-oxidative degradation of lipids (Golding *et al.*, 2010). The catechins, especially epigallocatechin gallate (EGCG), have been found to have more than 20 times the relative potency of vitamin E for preventing lipid peroxidation and more than 4 times the relative potency of butylated hydroxyanisole, which is often added in food to preserve fat or oil (Golding *et al.*, 2010). In addition, the catechins can inhibit the formation of mutagens, normally formed

during broiling or frying of meats, which have been shown to increase the risk of developing cancers such as breast and colon cancers (Golding *et al.*, 2010).

In the Asian food industry, the green tea catechins have been used in a wide range of commercial products as functional food ingredients to enhance both the shelf-life of products and to provide added health benefits for consumers (Golding *et al.*, 2010). For example, the tea catechins have been added to cereals, cakes, biscuits, ice cream, and other dairy products, confectionary products, instant noodles, fried snacks, sausages, and soft drinks (Golding *et al.*, 2010).

Crude green tea catechins powders were more effective than α-tocopherol and butylated hydroxyanisole in lard under conditions of the active oxygen method at 97.8°C (Matsuzaki *et al*., 1985). The antioxidant indexes of green tea extracts correlated with their EGCG contents based on induction time measured by the Rancimat method with a ternary mixture containing lecithin, tocopherols, and propylene glycol in chicken fat (Lunder, 1992). The ethanol extracts of green teas strongly inhibited the oxidation of canola oil at 100°C, as shown by measuring oxygen consumption, whereas extracts of black teas showed little or no antioxidant activity (Rangarsson *et al*., 1977). In addition, it was reported that the hot water extract of green tea also show high antioxidant activity measured using the conjugated diene method (Golding, 2010).

2.6. The development of oxidative rancidity in foods

Fats, oils and lipid-based foods deteriorate through several degradation reactions both on heating and on long term storage. The main deterioration processes are oxidation reactions and the decomposition of oxidation products which result in decreased nutritional value and sensory quality. The off-flavours that develop during lipid oxidation normally act as a warning that a food is no longer edible, although this does not apply to polyunsaturated lipid supplements taken in capsule form. Understanding of the mechanisms by which lipids deteriorate developed rapidly during the twentieth century. Autoxidation reactions commonly show an induction period, which is a period during which very little change occurs in the lipids. After the end of the induction period, oxidative deterioration of the lipids occurs much more rapidly. Off-flavours become most noticeable after the end of the induction period. Lipid oxidation is one of the major causes of quality deterioration in muscle foods following storage at refrigerated or frozen temperatures. Often seen in later stages of storage, quality losses are manifested through a variety of mechanisms (Allen and Hamilton, 1994).

The two major components involved in lipid oxidation are unsaturated fatty acids and oxygen. In this process, oxygen from the atmosphere is added to certain fatty acids, creating unstable intermediates that eventually break down to form unpleasant flavor and aroma compounds. Although enzymatic and photogenic oxidation may play a role, the most common and important process by which unsaturated fatty acids and oxygen interact is a free radical mechanism characterized by three main phases:

Initiation: Initiator (In) + RH → InH + R•

Propagation: R• + O2 → ROO•

ROO• + RH → R• + ROOH

Termination: 2ROO• → O 2 + ROOR ROO• + R• → ROOR

Initiation occurs as hydrogen is abstracted from an unsaturated fatty acid, resulting in a lipid free radical, which in turn reacts with molecular oxygen to form a lipid peroxyl radical. While irradiation can directly abstract this hydrogen from lipids, initiation is frequently attributed in most foods, including muscle foods, to reaction of the fatty acids with active oxygen species. The propagation phase of oxidation is fostered by lipid–lipid interactions, whereby the lipid peroxyl radical abstracts hydrogen from an adjacent molecule, resulting in a lipid hydroperoxide and a new lipid free radical. Interactions of this type continue 10 to 100 times before two free radicals combine to terminate the process. Additional magnification of lipid oxidation, however, occurs through branching reactions (also known as secondary initiation). The radicals produced will then proceed to abstract hydrogen from unsaturated fatty acids (Michael, 2001).

Initiation: The direct reaction of a lipid molecule with a molecule of oxygen is highly improbable because the lipid molecule is in a singlet electronic state and the oxygen molecule has a triplet ground state. To avoid this spin restriction, oxygen can be activated by any of the following three initiation mechanisms.

- Formation of singlet oxygen;
- Formation of partially reduced or activated oxygen species such as hydrogen peroxide, superoxide anion, or hydroxyl radical; and/or
- Formation of active oxygen–iron complexes (ferryl iron or ferric–oxygen–ferrous complex).

In addition, the oxidation of fatty acids may occur either directly or indirectly through the action of enzyme systems, of which three major groups are involved: microsomal enzymes, peroxidases, and dioxygenases, such as lipoxygenase or cyclooxygenase. Therefore, activated oxygen species are likely to be present in the food item even before it is harvested, not just produced during processing and storage.

Propagation: Propagation reactions form the basis of the chain reaction process and in general include the following.

- Radical coupling with oxygen: $R\bullet + O2 \rightarrow ROO\bullet$
- Atom or group transfer: $ROO\bullet + RH \rightarrow ROOH$
- Fragmentation: $ROO\bullet \rightarrow R\bullet + O2$
- Rearrangement and Cyclization

Conditions that determine the chain propagation length include initiation rate, structures of aggregates (increasing with increasing structure of the aggregates), temperature, presence of antioxidants, and chain branching. Chain branching involves the breakdown of fatty acid hydroperoxides to the lipid peroxyl or alkoxyl radical. Given the bond dissociation energies of LOO–H (about 90kcal/mol) and LO–OH (about 44kcal/mol), spontaneous decomposition is unlikely at refrigerated or freezing temperatures. Instead, breakdown of hydroperoxides would be dominated by one-electron transfers from metal ions during low temperature storage. The major contributors to decomposition of lipid hydroperoxides in food and biological systems would be heme and non-heme iron, with reactions involving the ferrous ion occurring much more quickly than those involving ferric ion.

Termination: To break the repeating sequence of propagating steps, two types of termination reactions are encountered: radical–radical coupling and radical–radical disproportionation, a process in which two stable products are formed from two radicals by an atom or group transfer process. In both cases, non-radical products are formed. However, the termination reactions are not always efficient. Secondary and primary peroxyl radicals, on the other hand, terminate efficiently by a mechanism in which the tetroxide decomposes to give molecular oxygen, an alcohol, and a carbonyl compound.

The retardation of these oxidation processes is important for the food producer and, indeed, for all persons involved in the entire food chain from the factory to the consumer. Oxidation may be

inhibited by various methods including prevention of oxygen access, use of lower temperature, inactivation of enzymes catalyzing oxidation, reduction of oxygen pressure, and the use of suitable packaging. Another method of protection against oxidation is to use specific additives which inhibit oxidation. These are correctly called oxidation inhibitors, but nowadays are mostly called antioxidants.

2.7. Antioxidants

A substance that, when present at a low concentration compared with that of an oxidizable substrate, inhibits oxidation of the substrate. Oxidation is a chemical reaction that transfers electrons from the substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions that deteriorate foods. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions, so that extends the induction time of the foods. They do this by being oxidized themselves, so antioxidants are often called reducing agents such as thiols, ascorbic acid or polyphenols.

Addition of antioxidants after the end of this period tends to be ineffective in retarding rancidity development. The induction time (IT) is very sensitive to small concentrations of components that shorten it; the pro-oxidants, or lengthen; antioxidants. Metal ions are the most important pro-oxidants in foods, whereas antioxidants include compounds that act by radical scavenging, metal chelating or other mechanisms (Halliwell and Gutteridge, 2007).

Antioxidants can inhibit or retard oxidation in two ways: either by scavenging free radicals, in which case the compound is described as a primary antioxidant, or by a mechanism that does not involve direct scavenging of free radicals, in which case the compound is a secondary antioxidant. Primary antioxidants include phenolic compounds such as vitamin E (α-tocopherol). These components are consumed during the induction period. Secondary antioxidants operate by a variety of mechanisms including binding of metal ions, scavenging oxygen, converting hydroperoxides to non-radical species, absorbing UV radiation or deactivating singlet oxygen. Normally, secondary antioxidants only show antioxidant activity when a second minor component is present. This can be seen in the case of sequestering agents such as citric acid

which are effective only in the presence of metal ions, and reducing agents such as ascorbic acid which are effective in the presence of tocopherols or other primary antioxidants (Michael, 2001).

The most important mechanism of antioxidants is their reaction with lipid free radicals, forming inactive products. Additives with this mechanism are antioxidants in the proper sense. Usually, they react with peroxy or alkoxy free radicals, formed by decomposition of lipid hydroperoxides. Other inhibitors stabilize lipid hydroperoxides, preventing their decomposition into free radicals. Some substances called synergists demonstrate no antioxidant activity in themselves, but they may increase the activity of true antioxidants (Allen and Hamilton, 1994).

2.8. Types of antioxidants

2.8.1. Natural antioxidant

Natural antioxidants are various substances with different chemical characteristics, which are widely present in plants. Antioxidants retard or inhibit oxidation of other substances by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu *et al.,* 1998). Consequently, natural antioxidants can protect both the biologically important cellular components and foods from oxidative processes caused by reactive oxygen species (Pamela, 2011).

Recently, food industries are obliged to include natural antioxidants in various products to delay oxidative degradation of lipids, improve quality and nutritional value of foods, and replace synthetic antioxidants (Pamela, 2011). Most natural antioxidants are common food components, and have been used in the diet for many thousands of years so that humans have adapted to their consumption (Jan and Michael, 2001).

2.8.2. Synthetic antioxidants

Synthetic antioxidants are a derivative of phenolic compounds and active as antioxidants, so only a few are used for food stabilization because of very strict safety regulation. Most of the approved antioxidants are phenolic derivatives, usually substituted by more than one hydroxyl or methoxy group. Among heterocyclic compounds containing nitrogen, only ethoxyquin is used (2, 6-dihydro-2, 2, 4-trimethylquinoline (Therisson *et al*., 1992), but now exclusively in feeds. Diludine (a substituted dihydropyridine derivative) is used for the stabilization of carotene and some pharmaceutical preparations, but not in food in spite of its good activity in fats and oils (Kourimská *et al*., 1993).

The common known synthetic antioxidants that used to retard or minimize oxidative deterioration of foods are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butyl hydroquinone (TBHQ) (Fasseas *et al.*, 2007).

2.9. General sources of antioxidants and their preparation

Herbs, spices and teas are one of the most important targets in the investigation of natural antioxidants from safety the point of view. In the evaluation of spices as natural antioxidant, some investigations have been carried out using the whole spice. Rosemary and sage were the most effective antioxidants in lard and both spices were found to have a low redox potential in sausages indicating antioxidative activity. However, in an oil-in-water emulsion, clove was the most effective spice. In general, the stabilization factors obtained for the spices in the emulsions were several times greater than those in lard, indicating a higher efficiency against oxidation in the emulsion (Jan and Michael, 2001).

The preparation of synthetic antioxidants and natural antioxidants for application in food products and processing are quite different. Synthetic antioxidants are produced as pure substances of constant composition, and are applied as such or in well-defined mixtures with other pure substances. Application is thus relatively easy, requiring no substantial modifications of the recipe and processing conditions. On the contrary, natural antioxidants are available from raw materials of variable composition. Both the content of active substances (usually a mixture of several compounds) and the content of various other compounds, either inactive or possessing negligible activities, depend on the plant variety, agro-technology, climatic conditions, degree of ripeness, and many other factors. Their composition should be determined in every batch, and if necessary, the procedure of their preparation or application, and the amount added to food products should be adapted according to analytical results. Some nature-identical antioxidants, such as α-tocopherol or β-carotene, are available on the market in a pure form or in defined solutions so that they can be added very easily in the amount desired. Solutions of these compounds are prepared in the industry in order to improve the solubility of the preparation in the food to be stabilized (Jan and Michael, 2001,).

2.10. Application of antioxidants

Antioxidants have a wide range of applications in different sectors. From many applications of antioxidants the following paragraphs discus the applications of antioxidants in food sectors and medicinal sectors.

2.10.1. Application of antioxidants in food industries

Lipids in foods of vegetable origin are usually more unsaturated than lipids of foods of animal origin, therefore, the initiation rate of oxidation reactions is higher and natural antioxidants, originally present in foods are more rapidly consumed than in lard or tallow and other animal fats. The stabilization of products of vegetable origin against autoxidation is thus less efficient than the stabilization of animal products. Protection factors of comparable antioxidants are several times higher in lard than in edible oils (Jan and Michael, 2001).

Edible oils become rancid on storage, the type of rancid off-flavor depending on their fatty acid composition (for example, it may become painty and fishy in oils containing linolenic acid, such as rapeseed oil) and the presence of minor components (for example, in flavor-reverted soybean oil). Edible oil producers try to prolong the shelf life of edible oils by different techniques, including the addition of antioxidants. The presence of natural antioxidants should always be taken into account, when appropriate levels of added antioxidants are considered (Mariassvoya, 2006).

The antioxidative effect of an ethanol extract from savory (*Satureja hortensis L*.) in sunflower oil was investigated during high temperature treatment (at 180°C). The extract improved the oxidative stability of sunflower oil even after 50 hrs at 180 °C and inhibited the oxidative processes more than the thermal processes under these conditions. Main components of the extract are thymol and carvacrol, the former being more active than the latter (Beddows *et al.*, 2000).

According to(Golding *et al.*, 2010), the catechins of green tea especially epigallocatechin gallate (EGCG), have been found to have more than 20 times the relative potency of vitamin E for preventing lipid peroxidation and more than 4 times the relative potency of butylated hydroxyanisole, which is often added in food to preserve fat or oil (Golding *et al.*, 2010). In addition, the catechins can inhibit the formation of mutagens, normally formed during broiling or

frying of meats, which have been shown to increase the risk of developing cancers such as breast and colon cancers (Golding *et al*., 2010).

2.10.2. Application of antioxidants in medicinal sectors

Antioxidants are widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer and coronary heart disease.

According to Duda-Chodak *et al. (2008),* catechins of green tea function as anticancer, antibacterial, antiviral, antitoxin and antifungal. Besides, it was proven that drinking too much tea that contains catechins would not affect human's life. In the pharmaceutical industry, the tea catechins have been used in toothpastes, mouthwashes, and breath fresheners to improve oral health (Golding *et al*, 2010). In addition, they have been used as supplement tablets or in drinks to enhance consumer health (Golding *et al*., 2010). They have also been added to air filters in ''antiinfluenza'' masks for protection from airborne viruses (Golding *et al*., 2010). In the cosmetics industry, the tea catechins have been used in various products, such as shampoos, moisturising creams, perfumes, and sunscreens with the aim of providing soothing effects on the skin as well as protecting the skin from free radical damage (Golding *et al*., 2010).

2.11. The regulation of antioxidants in foods

Since food is essential to life and can be improperly prepared or handled, it can threaten life. The purveyor of food therefore has a duty to provide safe and wholesome products to every customer. Given the fundamental importance of food, it is appropriate for any government to define and enforce this ethical obligation and thereby protect what many would consider the right of every individual to safe and wholesome food. From the legal point of view, antioxidants are substances which prolong the shelf-life of foodstuffs by protecting them against deterioration caused by oxidation, such as fat rancidity, color changes and loss of nutrient value. Antioxidants are extensively tested for the absence of carcinogenetic and other toxic effects in themselves, in their oxidized forms, and in their reaction products with food constituents, for their effectiveness at low concentrations, and for the absence of the ability to impart an unpleasant flavor to the food in which they are used (Jan and Michael, 2001).

Antioxidants should satisfy several requirements before being accepted for incorporation into food products. The use of antioxidants in food products is governed by regulatory laws of the individual country or by internal standards. Even though many natural and synthetic compounds

have antioxidant properties, only a few of them have been accepted as generally recognized as safe substances for use in food products by international bodies such as the Joint FAO Expert Committee on Food Additives and the European Community's Scientific Committee for Food. Antioxidants can be added directly to vegetable oils, melted animal fats or other fat-containing or polyphenols-containing systems. Food products can also be sprayed with, or dipped in solutions or suspensions of, antioxidants, or they can be packed in films containing antioxidants.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Experimental frame work of the thesis

The research was conducted based on the following general diagram which shows main unit operations and all activities performed during the work.

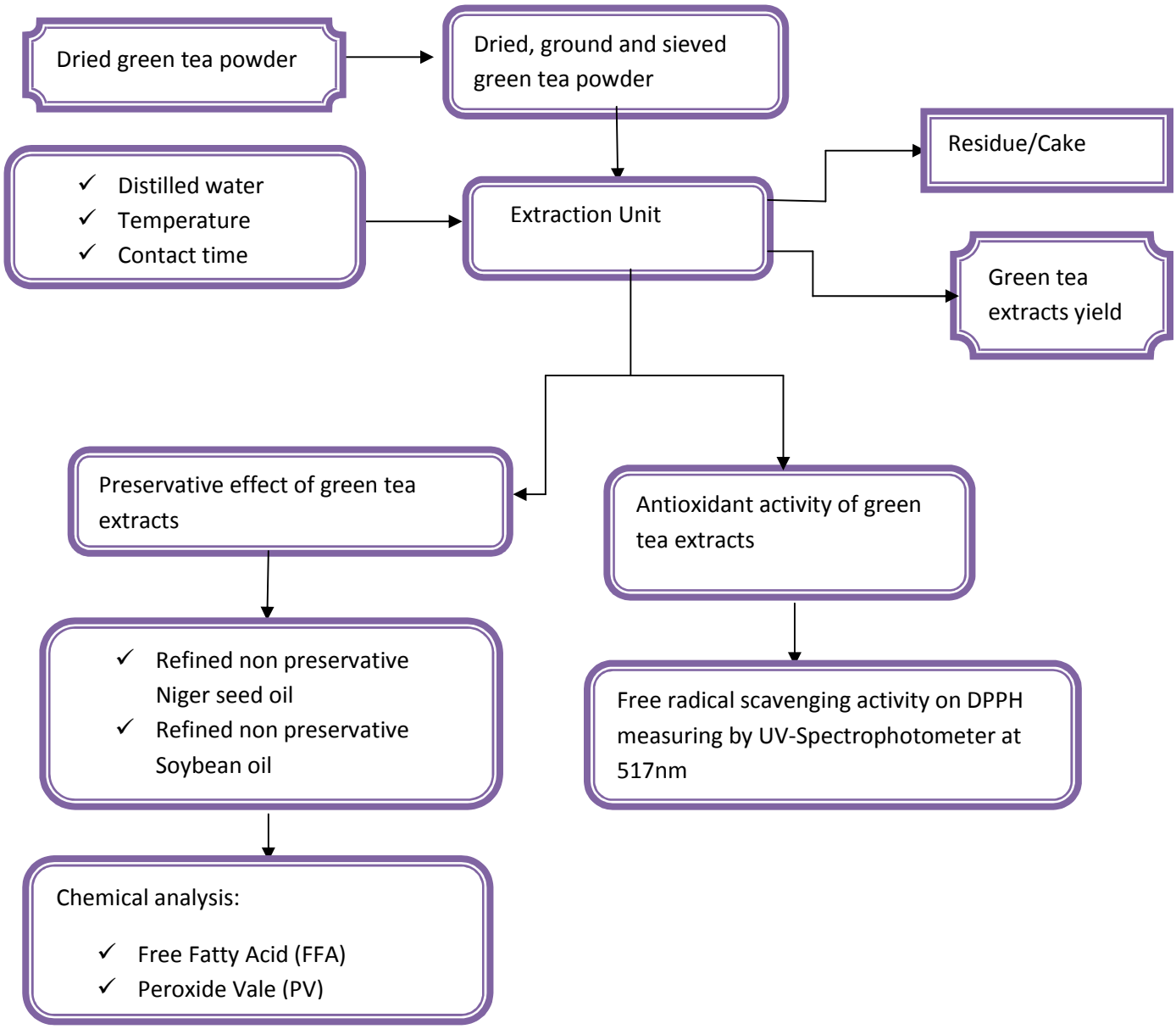


Figure 3.1. General Experimental Frame Works of the thesis

3.2. Sample collection and preparation

The experimental raw materials, *Camellia* leaves**,** were collected from Ethio Agri-CEFT tea farm located in Kaffa zone, Southern Nations Nationalities and Peoples Region. As the Camellia leaves harvested, it was boiled in hot water for 5 min to inactivate the enzyme. So that the leaves couldn't undergo oxidation process. Finally, the boiled Camellia leaves were packed by polyethylene bag and brought to School of Chemical and Bioengineering laboratory were the size reduction and other activities had been carried out.

3.3. Experimental Location

The experimental works of the thesis had been conducted mainly in three institutions. Size reduction of the raw and extraction process was conducted at the School of Chemical and Bioengineering, testing the potential antioxidant activity was conducted at Food Science and Nutrition department in 4kilo campus and the preservative effect of crude green tea extracts was conducted at Ethiopian Public Health Institute, Environmental and Nutrition department.

3.4. Materials and Equipment

3.4.1. Materials

Raw materials: Tea **(***Camellia sinensis***)** green leaves which are already dried, ground, sieved and packed were obtained from Ethio Agri-CEFT that found in Addis Ababa city. On the other hand, the food samples, Niger seed oil and Soybean oil were brought from the known company. Accordingly, the Niger seed oil was obtained from Abyssinia Niger seed oil PLC. While the Soybean oil was obtained from Fortune Company. Here, both oil samples are refined but non-preservative oils, synthetic antioxidants could not be included in it. Besides, the rationale behind that I choice these food samples are due to they are commonly used oil types by the community.

Chemicals: The chemicals that were used for the experiment are Acid chloroform, Potassium iodide (KI) solution, Sodium thiosulfate solution, Starch solution, distilled water (extraction solvent), and neutralized ethanol, phenolphthalein indicator, sodium hydroxide (NaOH), Ca_2SO_4. K_2SO_4, con.H_2SO_4, DPPH powder, methanol, Standard ascorbic acid. All analytically graded reagents are required.

3.4.2. Equipment

The equipment that were used for the experiment work were: Computerized UV-Spectrophotometer (517nm), condenser, sieves, 10ml volumetric flasks, vacuum filter, drying oven, Erlenmeyer flasks, small bottles (glass jars), sensitive balance, pH meter, measuring

beaker, quantitative filter paper which is equivalent to what man No. 42., Laboratory thermometer, spoon, pipette, micropipette, vortex mixer, test tubes, cuvettes (1cm, 2ml plastic or glass, racer, furnace, Kjeldahl flask.

3.5. Setting Extraction Parameters

For this thesis the independent variables or factors which have a direct effect on the dependent variables or response (yield and antioxidant activity) are extraction solvent (distilled water) concentration of 100 ,150 and 200 ml, extraction temperature (80 ,85 and 90 °C), and extraction time (10 ,35 and 60 min.) for actual variable levels. For each factors, an experimental range was adjusted based on the result of literature data. In order to determine conditions that optimized the extraction process as well as the response, the Face Centered Central Composite Design (FCCCD) with three central points were used.

These three factors: extraction solvent concentration of distilled water, extraction temperature and extraction time were selected as independent variables, because of their influence on antioxidant properties of phenolic extracts in plant materials (Wettasinghe and Shahidi, 1999).

In this study, the particle size was controlled as constant by passing through 500µm and retaining on 355µm sieve openings. The 355-500µm sieve size is optimal for extraction, while smaller particles may become slimy during extraction and create difficulty during filtration (Sukhdev *et al.*, 2008).

3.6. Aqueous Extraction of Antioxidants from Green Tea

Experimental Procedure: From the source, 10g of the ground green tea samples were extracted in 100,150 and 200ml hot distilled water at a temperature of 80, 85 and 90°C using water bath applying condenser to prevent water loss from the rounded flask, extractor for 60, 35 and 10 min, respectively. In such away, extraction was performed in duplicate. Finally, the crude extracts was cooled at room temperature and filtered by quantitative filter paper (what man No. 42) by applying vacuum filter. The filtered extract was then concentrated at 40°C and weighed to determine the yield (Aghamaamadi *et al*., 2011).

(a) Water Bath Extractor with condenser

(b) Water Bath extraction process

Figure 3.2. Water Bath Extractor and Extraction process.

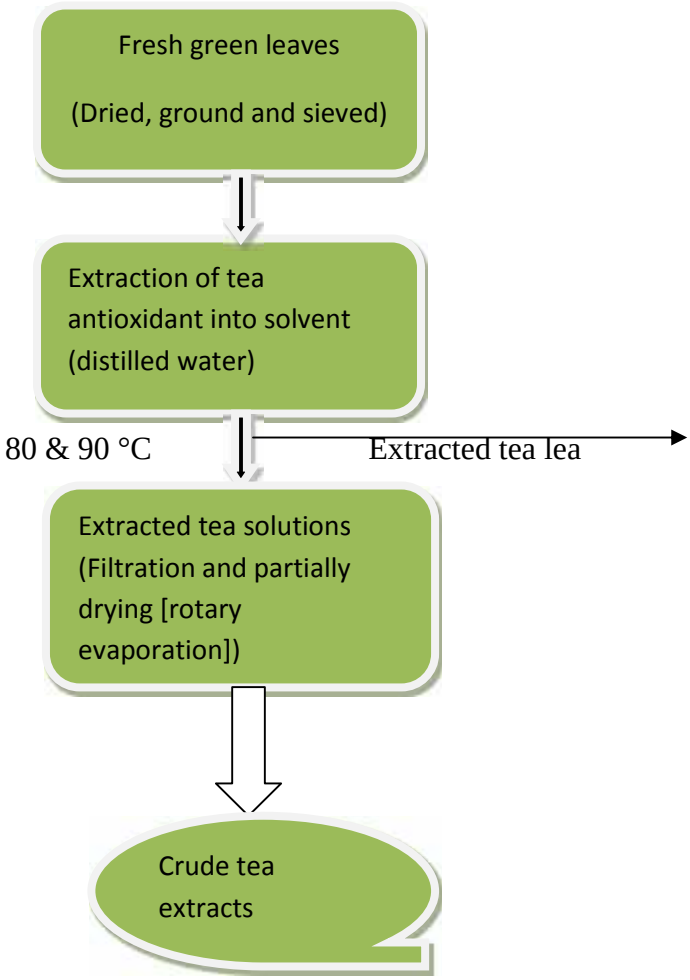


Figure 3.3. Extraction of antioxidant from green tea

3.7. Analysis Methods

The antioxidant activities of aqueous extraction of green tea leaves were evaluated and compared with the standard antioxidants, in this case standard ascorbic acid was used. In addition, the crude extracts were also subjected to pH, specific gravity and chemical composition determination.

3.7.1. Characterization of the extracts

3.7.1.1. pH value of the extract

The pH value of green tea extract was directly measured by pH meter at room temperature.

3.7.1.2. Color of green tea extract

After the Camellia leaves extracted in different experimental conditions the extract was cooled at room temperature and its color was measured using Uv- Spectrophotometer at the absorbance of 517nm.

3.7.1.3. Specific gravity of the extract

The sample was filled into graduated cylinder (50 ml) and its temperature was recorded. Hydrometer was used to measure the specific gravity of the green tea extract at the recorded temperature. Hence, the density of the green tea extract is determined using the specific gravity. Alternatively the following method was used.

Weight of beaker measured in (g)

Weight of green tea extract with beaker in (g)

Thus, Weight of green tea extracts

= Weight of green tea extract with beaker in (g) - Weight of beaker measured in (g)

Equal volume of water was measured.

Weight of water with beaker in (g)

Weight of water= Weight of water with beaker in (g) – weight of beaker (g)

Then, the specific gravity of the green tea extract was obtained by taking the ratio of green tea extract to mass of water.

3.7.1.4. Proximate analysis

Proximate analysis of green tea powder and extracts such as moisture content, ash content, protein, total carbohydrate, and crude fat were done using the method of Association of Official Analytical Chemists (AOAC, 2000).

Moisture content: About 2.0 g of green tea powder sample was weighed and placed in a crucible of constant weight. This was placed in an oven at 105℃ then dried; the weight was measured carefully to get a constant weight. The loss in weight indicates the moisture content of the sample.

*Ash content determination***:** Crucibles used for ash content determination was weighed and dried in an oven at 110℃ to a constant weight. About 2.0 g of the green tea powder sample was weighed and placed in the crucible then the weight of the crucible and sample was taken. This was placed in a furnace and ignited for 3hrs. at 550℃ till the sample has a cotton wool like texture; it was cooled in a desiccator and weighed using analytical balance.

Protein: About 1.0 g of the sample was weighed into the Kjeldahl flask. About 0.1 g of Ca_2SO_4 and 1.0 g K_2SO_4 were added into the flask with 20ml of concentrated H_2SO_4**.** The flask was then placed in a slanting position on the Kjeldahl digestion heating mantle in the furne cupboard. Digestion continual until there was a color change which was from black to bluish-green signifying that digestion has ended. It was set up against blank, the digest were removed and allowed to cool and was then diluted with water and made up to 200ml on ice.

About 50ml of aliquot of digest was poured into a distillation flask. About 50ml of NaOH were carefully layered into the solution in order to make it a strong alkaline and 50ml of 0.1N H_2SO_4 measured and kept in a beaker with two drops of methyl red as an indicator. The H_2SO_4 acted as a receiving flask.

About 150ml was distilled over then distillation was stopped by removing the solution in the receiving flask before the heat was put off to avoid drop in pressure. The distillate (excess acid) was treated with 0.1 M NaOH in the burette. This was done for both the sample and the blank and Ended, the percentage of nitrogen was calculated.

Lipid: About 1.0 g of the sample was weight into a thimble of known weight. About 150 ml pet ether (60-80) ℃ were poured into 250 ml conical flask using the measuring cylinder. The soxhlet extractor where the sack and its content had been introduced was fitted and the solvent boiled under reflux. The extraction processed lasted for about 8 hrs. , the sack with its content was removed, dried in an oven for 2 hours and then weighed with an analytical balance.

Carbohydrates: The total carbohydrate (%) of the sample by mass including crude fiber was obtained according to Medwell journals, 2011.

Total carbohydrate (%) = 100 - [M+A+P+L]

Where:

M = moisture content % A = ash content %

P = protein % L = lipid %

3.7.1.5. Evaluation of green tea antioxidant activity

The best combination of extraction parameters such as extraction temperature, time and solvent concentration for maximum green tea extract antioxidant activity and yield were taken for further antioxidant activity evaluation and its preservative effect study. The Antioxidant activity of green tea extract was determined by UV/ visible light spectrophotometer using DPPH (1, 1 Diphenyl-1-Picrylhydrazyl) free radical scavenging activity.

3.7.1.5.1. Determination of free radical scavenging activity

This method is based on the reduction of stable DPPH when it accepts hydrogen from an antioxidant compound. Radical scavenging activity of distilled water extracts from green tea powder against stable DPPH was determined spectrophotometrically. The changes in color (from deep-violet to light-yellow) were measured at 517nm using a UV-visible light spectrophotometer.

Reagents and solutions preparation: deionized or distilled water was used for all recipes and protocol steps. 0.01g of 0.004% DPPH was taken and dissolved in 250ml methanol in volumetric flask. Indeed, 0.075g of standard ascorbic acid was measured and dissolved in 1ml of methanol in 25ml of volumetric flask for comparison.

Procedure: the hydrogen atoms or electrons donation ability of the extracts and some pure compounds were measured from the bleaching of purple colored methanol solution of DPPH. The effect of distilled water extracts on DPPH radical was estimated according to Kirby and Schmidt (2004). Briefly,

About 4ml of 0.004% solution of DPPH radical solution in methanol was mixed with 1ml of various concentrations (20, 40, 60, 80, 100 and 120µl) of the extracts in methanol with a vortex mixer (Appendice B). Samples were incubated for 30min in the dark at room temperature. The scavenging capacity was read spectrophotometrically by monitoring the decrease in absorbance at 517 nm using a UV-vis spectrophotometer. Finally, inhibition of free radical DPPH in percent (%) was calculated in following way:

$$\%\text{Inhibition} = [(A_B - A_A]/A_B\ ] \text{ X } 100$$

Where:

A_B –absorption of blank sample (t= o min)

A_A – absorption of extract sample (t= 30 min)

Estimation of the scavenging activity was carried out in duplicate. The results were reposted mean/ average of the two parallel measurements.

(a) Computerized UV-Spectrophotometer

(b) Spectrophotometric antioxidant activity Evaluation

Figure 3.4.Show photo of computerized UV-spectrophotometer and antioxidant activity evaluation of green tea extracts on DPPH.

3.8. Preservative effect of green tea extracts on soybean and Niger seed oils

Green tea extract was added to each test samples of soybean and Niger seed oils at three different concentration levels: 0, 0.1 and 0.2% and the samples were stored for 7, 14 and 21 days, according to the literature (Gebrehana and Shimelis, 2013). A total of nine samples for each soybean and Niger seed oils were prepared. Each has a weight of 40g and treated with 0, 0.1 and 0.2% crude green tea extracts. The samples were stored at room temperature in dark place for three consecutive weeks. The rancidity parameters such as free fatty acid, peroxide value and acid value were evaluated for each treated samples per the storage weeks.

Determination of Free Fatty Acids

Free fatty Acid of soybean and Niger seed oils were determined according to (AOAC, 2003) Official method of 972.28.

About five gram of melted each oil samples were placed in 250ml Erlenmeyer flasks and 100ml of neutralized ethanol and 2ml of phenolphthalein indicator were added. The mixture was vigorously shake and titrated with standard 0.1N NaOH base until the endpoint reached when slight pink color can persist for 30 seconds. This was done in duplicate and the volume of the titrant was recorded for acid value/free fatty acid calculation. Similarly, blank was prepared without adding oil samples and the amount of titrant it took was recorded. For each oil samples, the free fatty acid was calculated as the following formula.

$$\%FFA \text{ (as oleic acid)} = \frac{V*N*28.2}{W}$$

Where:

%FFA = % of free fatty acid expressed as oleic acid

V = volume of NaOH titrant (ml)

N = normality of NaOH titrant (mol/1000ml) and

W = sample weight

Determination of Peroxide Value

The Peroxide value was evaluated according to AOCS Official Method Cd 8-53 (2003).

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

$$PV = \frac{10\,X\,(v1-v2)}{m}$$

Where: V1 =volume of $Na_2S_2O_3$ for determination of test samples in ml,

V2 =volume of $Na_2S_2O_3$ for determination of blank solution in ml and

m = mass of test portion in g (5g)

3.9. Experimental design and Statistical analysis

In this study, the Face Centered Central Composite Design (FCCCD) under the response surface methodology was used to determine the influence of extraction solvent concentration, extraction

temperature and extraction time on the yield and antioxidant activity of green tea and to identify the optimum levels. The three independent variables (solvent concentration, temperature and time) were investigated at three levels as shown in Table 3.6.

Table 3.1 Different level of factors associated with the experiment.

Factors	Low (-1)	Medium (0)	High (+1)
Extraction solvent con.	100ml	150ml	200ml
Extraction temperature	80 ℃	85℃	90℃
Contact time	10 min.	35min.	60min.

The detail experimental designs were presented in 3.6.2. Seventeen experiments with three replications at the center points were studied. A 2k factorial design gave a total of 17 experimental runs.

Runs = 2^k + 2k +n_c = 2^3 +2(3) +3 = 8 +6 +3 = 17

Where k is the factors and n_c is center point.

Table 3.2. The Face Centered Composite Design Experiment Performed for Green tea extracts**.**

Run	Factors			Responses	
	Extraction Solvent concentration (ml)	Extraction temperature (℃)	Extraction time (min.)	Yield of green tea extracted (ml)	Antioxidant activity of green tea
1	200(+1)	90(+1)	60(+1)		
2	200(+1)	80(-1)	60(+1)		
3	150(0)	90(+1)	35(0)		
4	200(+1)	85(0)	35(0)		
5	100(-1)	90(+1)	10(-1)		
6	150(0)	85(0)	10(-1)		
7	150(0)	85(0)	35(0)		
8	100(-1)	80(-1)	60(+1)		
9	200(+1)	80(-1)	10(-1)		
10	150(0)	80(-1)	35(0)		
11	200(+1)	90(+1)	10(-1)		
12	100(-1)	90(+1)	60(+1)		
13	100(-1)	80(-1)	10(-1)		
14	150(0)	85(0)	60(+1)		
15	100(-1)	85(0)	35(0)		
16	150(0)	85(0)	35(0)		
17	150(0)	85(0)	35(0)		

The statistical software package Design Expert version 7.0.0 (Stat Ease Inc., Minneapolis, USA) was used to generate the experimental data and develops the regression model. The quality of fit of the regression model expressed as the coefficients of determination (R^2), the statistical significance determined by ANOVA the response surface and the contour plots were all study to estimate the models as well as to determine the optimum levels.

3.9.1. Face Centered Central Composite Design (FCCCD)

Central composite design is widely used for fitting a second order response surface. It involves use of a two-level factorial combined with 2k axial points. As a result, the design involves, say, F factorial points, 2k axial points, and n center runs. The factorial points represent a variance-optimal design for a first order. Center runs provide information about the existence of curvature in the system. If curvature is found in the system, the addition of axial points allow for efficient estimation of the pure quadratic terms. In some real experimental setup the region of design is a cube. In such scenario, a central composite design in which eight corners of the cube are centered and scaled to (±1, ±1, ±1). This type of design is known as Face Centered Central composite design (Fig.1).the face centered central composite design is useful for the experiments when there is need to fit a second order response surface (Montgomery ,2001).

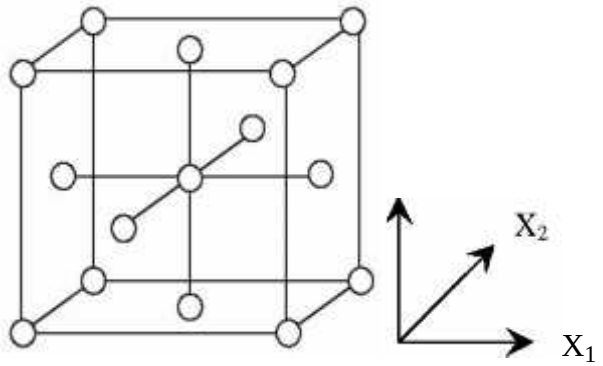


Figure3.5. Geometry of Face Centered Central Composite Design

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1. Characterization of green tea extracts

In this study, the characterization of green tea extracts such as pH, specific gravity in relative to water and proximate analysis had been conducted. After the green powder of green tea was extracted by distilled water and cooled at room temperature, the pH was measured by pH meter and obtained the value as 6.6. While the color of the extract was 0.028 as measured by Uv-Spectrophotometer. The specific gravity of the extract was calculated based on the measured values of empty beaker and samples (extracts) as well as water with the same amount to that of the extracts as stated in section 3 of this manuscript. Thus, the specific gravity of the extracts = mass of water/ mass of extracts, which is 0.864. Specific gravity is the heaviness of a substance compared to that of water, and it's expressed without units.

The proximate analysis of green tea extracts such as moisture content, ash content, protein, lipid and total carbohydrate including crude fibers were determined based on the procedure which was stated under the materials and methods of this manuscript. Below is the table which shows the summary of the proximate analysis values.

Table 4.1. Proximate analysis of green tea extracts

Proximate analysis	Contents (%)
Moisture content	11.8 ±0.55
Ash content	4.7 ±0.01
Protein	6.4.0 ±0.00
Lipid	5.5 ±0.01
Total carbohydrate	70.0 ±0.00

Experiments were done in duplicate and all values are in mean ±SD

4.2. Effect of extraction parameters on yield (response) of green tea extracts

In this study the effect of extraction parameters (solvent extraction, extraction temperature and extraction/contact time) on both yield and antioxidant activity of *Camellia sinensis* (green tea)

extracts were studied. Table 4.2. Showed the effect of extraction parameters on green tea extract yield. Yield of green tea extracts was determined on the basis of input-output results after the extracts were concentrated in water bath at the temperature of 60℃. Table 4.1 shows that distilled water concentration has direct relation with the yield of green tea extracts; it increases with the distilled water concentration increases. Based on the result one-way ANOVA analysis, it has significant ($p < 0.05$) positive effect on green tea extract yield. The highest (78.3 %) and the lowest (52.6%) yield of extracts were obtained at 200 and 100 ml of distilled water concentrations, respectively. This highest yield extracts was obtained at a temperature of 90℃ with 60 min, which is exactly agree with the work of (Lin et al., 2008) that is extraction of tea components with hot distilled water at different temperatures for varying lengths of time and reported that the maximum yield of extracts was obtained at a temperature of 90℃ with a 60min extraction time. He also reported that the yield of the extract was found to decrease when the extraction was performed at temperatures lower than 90℃.

Table 4.2. FCCD experimental design showing the effect of extraction parameters on yield of green tea extracts input-output based.

Run	Extraction parameters			Yield of green tea extracts (%)
	Solvent con.(ml)	Extraction temp.(℃)	Contact time (min)	
1	200	90	10	76.2
2	200	80	10	70.8
3	200	80	60	72.6
4	100	90	10	58.5
5	150	85	10	62.5
6	150	85	35	69.7
7	150	85	35	69.7
8	150	90	35	68.5
9	200	85	35	74.7
10	200	90	60	78.3
11	150	85	60	67.2
12	100	85	35	55.8
13	100	80	10	52.6
14	150	85	35	69.7
15	150	80	35	62.0
16	100	80	60	53.8
17	100	90	60	60.0

All experiments were done in duplicate and results were put in average.

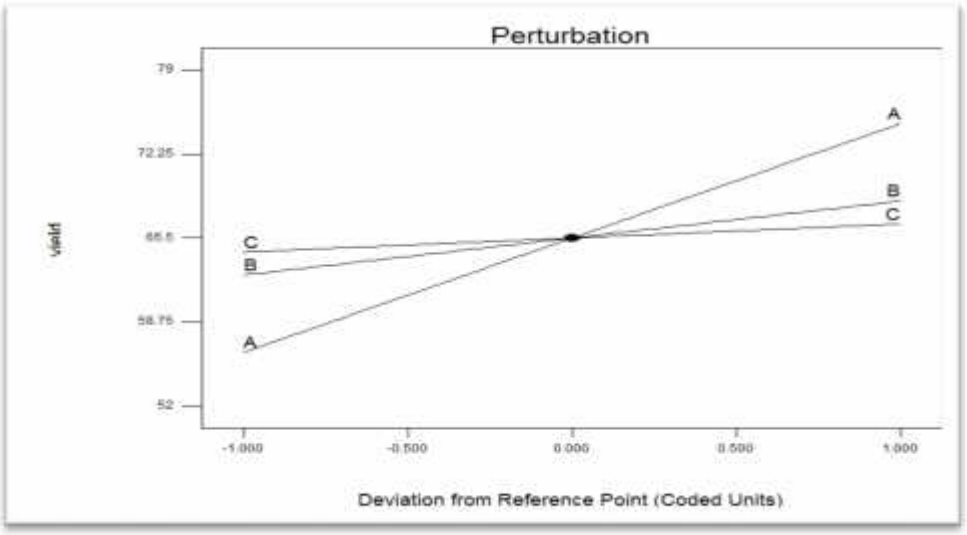


Figure 4.1. Perturbation graphs showing the interaction of factors

As it is observed from this graph, as each parameter increases and passes the reference point the yield also increases. Especially, the yield is highly increased with the increasing of extraction solvent, distilled water and temperature. This is because high extraction temperature improves extraction efficiencies due to heat renders the cell walls more permeable to solvents and components and increases the solubility and diffusion coefficients of the tea components to be extracted. However, excessive extraction temperatures above 80-90℃ can cause degradation of the catechins by promoting the change in epistructured catechins to non-epistructured catechins (Chen *et al*., 1998).

4.2.1. Validation of the experimental model for yield of green tea extracts

Table 4.1 summarizes the result obtained with the experimental design which was aimed in determining the conditions that favors maximum yield increase in green tea extracts. A linear and 2FI model equation (4.1 and 4.2) shown below were fitted to the data model for predicting response; yield of green tea extracts.

Yield (Y_1) = 65.52+9.19*A+2.97*B+1.13*C……4.1

Yield(Y_2) = 65.62 + 9.19*A+2.97*B+1.13*C-0.13*A*B+0.15*A*C+0.075*B*C……..4.2

Where A is extraction solvent concentration (distilled water), B is extraction temperature, and C is extraction/contact time, whereas the number 65.52 is the intercept term. These two model gave the same low probability P_{model} > F= 0.0001 and P_{model} > F=0.0001 when tested by Analysis of variance (ANOVA) Table 4.2. The significant lacks of fit of the two models indicate that the

model equations showed a close fit with the experimental results. The goodness of fit was evaluated by the coefficients of determination (R^2), which was 0.9758 and 0.9762, which are almost the same coefficients of determination and this reveals that 97.58% and 97.62%, of the data was explained by the two selected models. The adequate precision of 40.803 and 27.256 for linear and 2FI model respectively for green tea extract yield were greater than 4, which indicates the two models could be used to investigate the design space.

From the ANOVA analysis, (Table 4.2 a and Table 4.2b) for yield of green tea extracts as response, both linear and 2FI all extraction parameters, solvent concentration [A] (distilled water), extraction temperature [B] and extraction time[C] were significant ($P<0.05$). This emphasized that extraction parameters are a critical variables that influence the yield of green tea extracts. On the other hand, none of the interactive terms show significant effect ($P>0.05$) for 2FI model. The quadratic model also shows significant effect like the two models for the extraction solvent, but not for the interactive effect. However, the cubic model effect for the interactive term between extraction solvent concentration and contact time (AC) and extraction solvent concentration, extraction temperature and contact time (ABC) for response yield increase were found to be highly significant ,even though the model is aliased. This observation indicates that interactive effects are important for true optimization rather than the one factor at a time (OFAT) method.

Table 4.3a. Analysis of variance (ANOVA) for response surface linear model for yield.

Source	Sum of squares	Mean square	F value	Prob.>F	
Model	945.54	315.18	174.76	0.0001	significant
A	844.56	844.56	468.29	<<0.0001	
B	88.21	88.21	48.91	<< 0.0001	
C	12.77	12.77	7.08	0.0196	
Residual	23.45	1.80			
Lack of Fit	10.52	0.96	0.15	0.9878	not significant
Pure Error	12.93	6.46			
Cor Total	968.98				

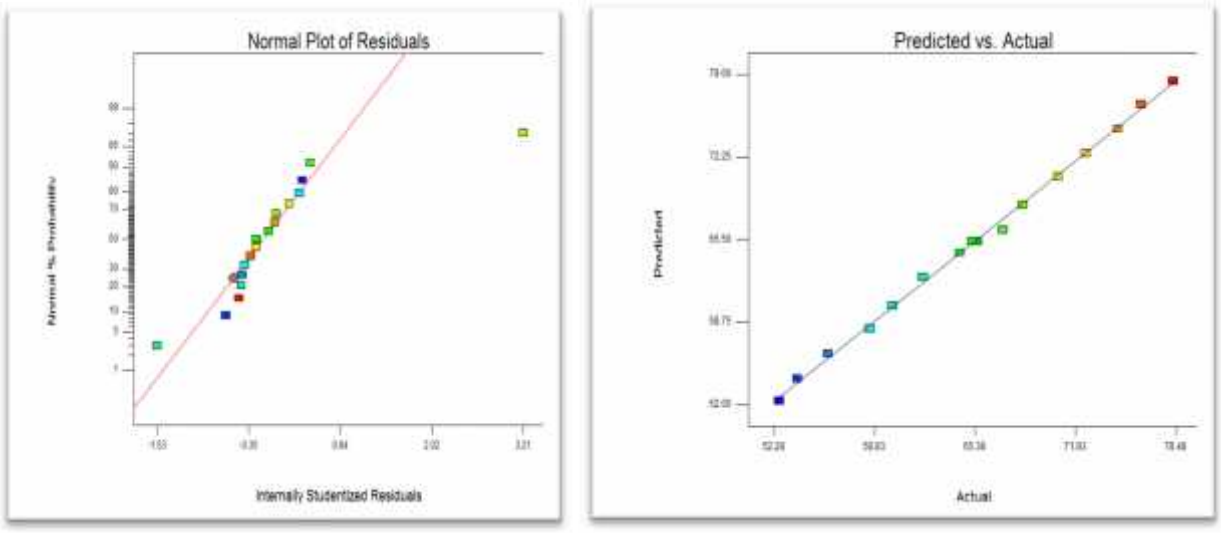
P< 0.05 indicates the model terms are significant while P<0.01 is highly significant

Table 4.3b.Analysis of variance (ANOVA) for response surface 2FI model for yield.

Source	Sum of squares	Mean square	F value	P>F	
Model	945.89	157.65	68.26	0.0001	significant
A	844.56	844.56	365.68	<<0.0001	
B	88.21	88.21	38.19	<< 0.0001	
C	12.77	12.77	5.53	0.0405	
AB	0.13	0.13	0.054	0.8207	
AC	0.18	0.18	0.078	0.7858	
BC	0.045	0.045	0.190	0.6653	
Residual	23.10	2. 31			
Pure Error	12.93	6. 46			
Lack of Fit	10.71	1.27	0.20	0.9624	not significant
Cor Total	968.98				

P< 0.05 indicates the model terms are significant while P<0.01 is highly significant.

Alternatively, Figure 4.1. Cheeked the adequacy of the selected model for yield of green tea extracts.



(a) Normal plot of Residuals (b) Predicted vs. Actual

Figure 4.2. Shows different model adequacy checking graphs for yield of green tea extracts.

4.3. Free radical scavenging activity and antioxidant activity of green tae extracts

The results obtained from DPPH assay for antioxidant activity of green tea extracts for selected run and concentration of the extracts based on the formula stated in section 3.4 is presented in Figure 4.3. The effect of extracts concentrations on antioxidant activity could be explained by comparing the scavenging activity for each runs after diluting the extracts with distilled water in the ration of 1:25ml to reduce the color effect on DPPH absorption of UV-Spectrophotometer. Table 4.4 shows the effect of extracts concentrations on antioxidant activity.

Table 4.4. Effect of green tea extracts concentrations/doses on antioxidant activity/% scavenging activity.

	Extracts concentrations (10^{-6} l)						Antioxidant activity of
Run	20	40	**60**	80	100	120	Ascorbic acid at 120 µl dose
1	93.6	92.9	**92.6**	92.9	93.1	92.5	95.5
2	29.6	57.0	**89.9**	94.9	95.2	95.3	95.0
3	33.3	55.8	**90.9**	92.6	93.0	93.2	94.8
4	11.6	44.2	**80.0**	90.8	79.3	87.5	95.6
5	6.5	24.8	**81.2**	55.9	66.8	77.9	96.8
6	55.8	39.9	**83.5**	89.8	92.7	91.9	90.0
7	55.8	39.9	**84.5**	89.8	92.7	91.9	90.0
8	46.2	68.1	**89.3**	93.8	93.8	93.9	95.4
9	51.8	82.5	**91.9**	83.8	84.2	83.9	94.4
10	33.6	69.3	**96.1**	91.8	91.6	91.6	92.0
11	28.5	47.9	**85.8**	81.7	87.4	89.6	95.8
12	41.6	72.8	**79.5**	93.5	93.7	93.4	95.8
13	34.7	65.4	**78.0**	91.8	90.6	91.2	95.3
14	55.8	39.9	**83.5**	89.9	92.7	91.9	90.0
15	53.1	85.0	**80.0**	92.1	90.8	91.7	94.0
16	56.8	94.0	**78.8**	96.5	95.9	95.8	95.0
17	32.2	67.5	**81.0**	94.3	80.0	94.7	94.9

All experiments were done in duplicate and results were kept in average

As it is observed from the table the antioxidant activity is somewhat increased as the concentration of the extracts is increased in almost all runs. This increment of antioxidant activity is highly observed from 20 microliter up to 60microliter of the extracts, but beyond 80 microliter the antioxidant activity is almost constant from this experimental view of point. Thus, for this experimental work values with 60 microliter ($6x10^{-5}$) l concentration of extracts which are written in bold color was used for antioxidant activity comparison between the runs in

addition to the extraction parameters. Therefore, as it's seen clearly from the above table in column 3, the highest value or the highest antioxidant activity (**95.1 %**) was registered run 10. This will be briefly discussed in the next section under effect of extraction parameters on antioxidant activity.

On the other hand, Ascorbic acid, the standard synthetic antioxidant, scavenging activity based on DPPH assay is almost constant for all runs at maximum concentration. Therefore, this used as a standard to evaluate the antioxidant capacity of the green tea extracts which is obtained by varying the extraction parameters. Figure 4.3 shows the antioxidant power comparison of distilled water extracts of green tea powder and standard Ascorbic acid measured by Uv-spectrophotometer on DPPH.

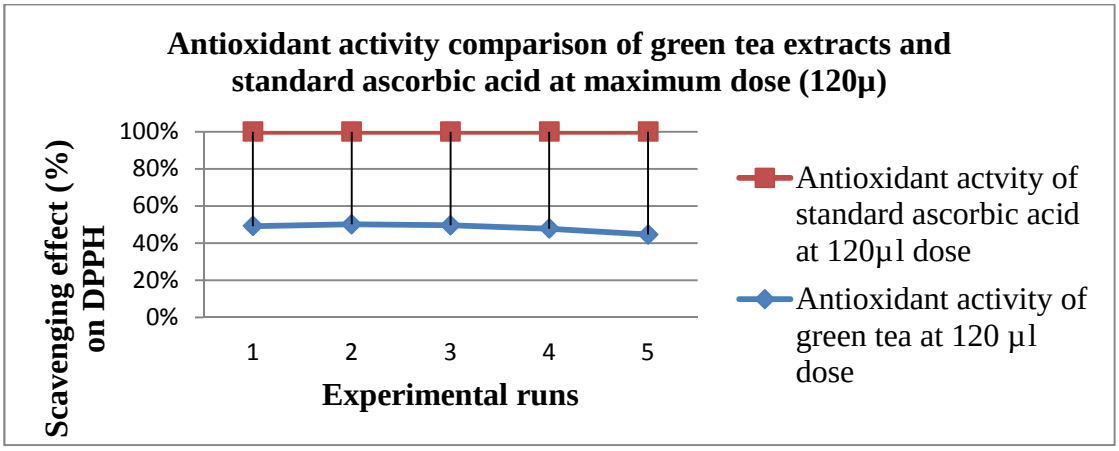


Figure 4.3. Scavenging of distilled water extracts of green tea and standard ascorbic acid on DPPH

Now, let's see whether the mean differences of green tea extracts antioxidant activity and standard ascorbic acid antioxidant activity is significance or not based on **paired t- test**. Table 4.5 shows comparison of mean difference significance of green tea antioxidant activity and standard ascorbic acid antioxidant based on paired t-test method.

Table 4.5 paired t –test comparison of green tea extracts and standard ascorbic acid antioxidants activity at 120µl dose.

Runs	Green tea antioxidant	Standard ascorbic acid antioxidant	Mean differences
1	92.5	95.5	-3.0
2	95.3	95.0	0.3
3	93.2	94.8	-1.6
4	87.5	95.6	-8.1
5	77.9	96.8	-18.9
6	91.9	90.0	1.9
7	93.9	95.4	-1.5
8	83.9	94.4	-10.5
9	91.6	92.0	-0.4
10	89.6	95.8	-6.2
11	93.4	95.8	-2.4
12	91.2	95.3	-4.1
13	91.7	94.0	-2.3
14	95.8	95.0	0.8
15	94.7	94.9	-0.2

Let μ_1= mean of green tea extracts antioxidant activity and μ_2= mean of standard ascorbic acid antioxidant activity. Then the statistical hypothesis test is conducted as follows.

Testing H_o: $\mu_1 - \mu_2$ is equivalent to testing

$H_o : \mu_d = 0$

$H_1: \mu_d \neq 0$

The computed value of the paired t-test statistic is

$t_o = \frac{\bar{d}}{Sd/\sqrt{n}}$ see the details (Montgomery,2001).

After all calculations are performed and suppose $\alpha = 0.05$ the following values are obtained:

$\bar{d}$ = -3.4467 $|t_o| = 1.980$

$S_d = 6.640$ $t_{0.025,14} = 2.145$

and because $t_{0.025,\ 14} = 2.145 > |t_o| = 1.980$, the hypothesis $H_o : \mu_d = 0$ could not be rejected. That is, there is no evidence to indicate that the green tea extracts and standard ascorbic acid have significance difference antioxidant activity.

4.4. Effect of extraction parameters on antioxidant activity of green tea extracts

The effect of extraction parameters on antioxidant activity of the extracts were studied by setting each parameters, that is extraction solvent concentrations (100, 150, and 200ml), extraction temperature (80, 85 and 90 ℃) and extraction/contact time (10, 35 and 60min.) as shown in Table 4.5. As it is observed from this table the lowest antioxidant activity was obtained at lower temperature (80℃), lower extraction solvent concentration (100ml) and at higher contact time (60 min.). Based on the result one -way ANOVA analysis, contact time and the interactive term (extraction solvent concentration and extraction temperature) have a significant effect ($P<0.05$) on the antioxidant activity of the extracts.

The highest antioxidant activity of the extracts that was obtained at a temperature of 90℃ with a 60min was also agree with the work of Lin et al., (2008) that was reported as extracting green tea powder at a temperature of 90 at 60min not only have high yield but also possessing greater antioxidant activity. But, increasing extraction temperature beyond 90, for more than 60min, phenolic compounds of green tea extracts could be degraded and resulted in the loss of its antioxidant activity. According to Chan *et al*. (2009); Liyana-Pathirana and Shahidi (2005), the loss in antioxidant capacities of plant extracts at high extraction temperature was likely due to the degradation of phenolic compounds which were previously mobilized at low temperature. Similarly, (Mueller-Harvey (2001) reported that some phenolic compounds decomposed rapidly under high temperature and thus caused a reduction in the antioxidant capacity of plant sample. Thus, it has been shown that the phenolic compounds which were extracted beyond 80 had relatively lower antioxidant capacity.

Table 4.6. FCCD experimental design showing the effect of extraction parameters on antioxidant activity of green tea extracts.

Runs	Extraction parameters			Response (Y_a) Antioxidant Activity (%)
	Solvent Con.(ml)	Extraction temperature (℃)	Contact time (min)	
1	200.0	90.0	10.0	92.6
2	200.0	80.0	10.0	89.9
3	200.0	80.0	60.0	90.9
4	100.0	90.0	10.0	80.0
5	150.0	85.0	10.0	81.2
6	150.0	85.0	35.0	83.5
7	150.0	85.0	35.0	84.5
8	150.0	90.0	35.0	89.3
9	200.0	85.0	35.0	91.9
10	200.0	90.0	60.0	96.1
11	150.0	85.0	60.0	85.8
12	100.0	85.0	35.0	79.5
13	100.0	80.0	10.0	78.0
14	150.0	85.0	35.0	83.5
15	150.0	80.0	35.0	80.0
16	100.0	80.0	60.0	78.8
17	100.0	90.0	60.0	81.0

All experiments were done in duplicate and results were kept in average

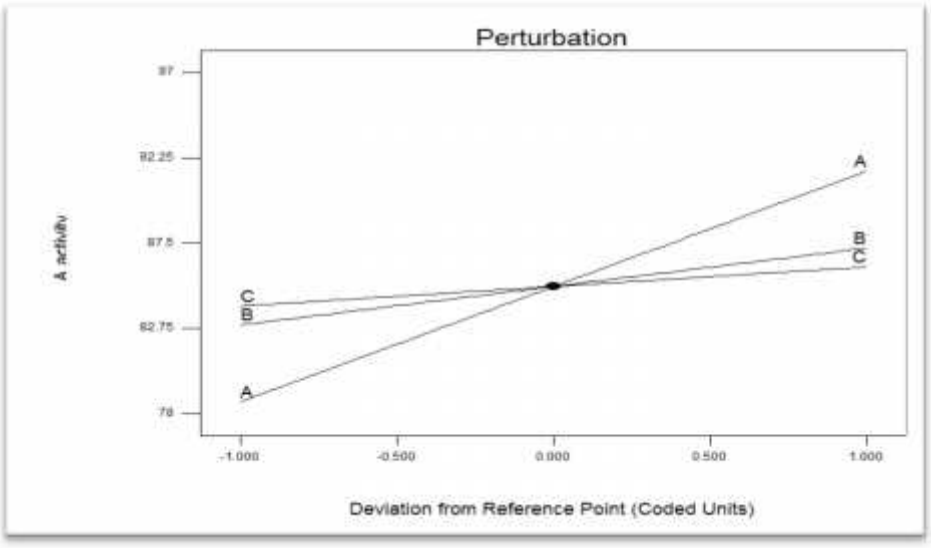


Figure 4.4. Perturbation graphs showing the interaction effect of factors.

It is clear that as the three extraction parameters (solvent con., extraction temp. and contact time)

increase and passé the reference point so does the antioxidant activity Figure (4.4.). Here, though the antioxidant property increases with temperature and contact time but decrease beyond the maximum value. Antioxidant activity of the extracts is more likely high up to the temperature of 90℃ and contact time 35 min as observed during the experimental session. This is because extracting antioxidant at a temperature of 95℃ with contact time of 20min improves antioxidant activity while decreases when decreases 90℃ (Lin et al., 2008).

4.4.1. Validation of the experimental model for antioxidant activity

Table 4.6 summarizes the result obtained with the experimental design which was aimed in determining the conditions that favors maximum antioxidant activity in green tea extracts. A second order quadratic model and linear equation which are shown below were fitted to the data model for predicting response; antioxidant activity.

Antioxidant activity (Y_a) = +85.09+6.41*A+2.14*B+1.09*C…….4.3

Antioxidantactivity(Y_a=+83.89+6.41*A+2.140*B+1.09*C+0.46*A*B+0.340*A*C+0.340*B*C+1.76*A^2+0.71*B^2-0.44*C^2……4.4

Where A is extraction solvent concentration, B is extraction temperature and C is extraction/ contact time. These model gave a low probability of P> F =0.0001 and P>F = 0.0005 for linear and quadratic model of the above two models, respectively for antioxidant activity when tested by Analysis of variance (ANOVA) as shown in Table 4.6. The significant lacks of fit of these models indicate that the model equation showed a close fit with the experimental results. The goodness of fit was evaluated by the coefficients of determination (R^2), which were 0.920 and 0.9590 for linear and quadratic model respectively. This revealed that 92.0% and 95.90 %, of the data were explained by the two selected models for antioxidant activity when compared to the model equation of yield of green tea extracts. The adequate precision of 22.458 and 14.557for linear and quadratic models of the antioxidant activity were greater than 4, which indicates the model could be used to navigate the design space. From the ANOVA analysis, (Table 4.5) for antioxidant activity as response, the quadratic effect of Extraction temperature (B) and Extraction time (C) were significant (P<0.05) while the quadratic effect of extraction solvent concentration was not significant (P>0.4926). However, the interactive term (AB) of extraction solvent concentration and extraction temperature show a significant effect (P<0.05) But, not the interactive of (BC) show a significant effect (P>0.05). This implies that a slight variation in concentration of the individual variables, extraction

temperature (B) and contact time (C) could bring change on antioxidant activity of the extracts. On the other hand, the square terms (A^2, B^2 and C^2) of all extraction parameters show significant effect (P>F=0.0040), (P>F=0.0026) and (P>F=0.0013) respectively.

Table 4.7a.Analysis of variance (ANOVA) for response surface linear model for antioxidant activity of the extracts.

Source	Sum of squares	Mean square	F value	P>F
Model	468.56	156.19	49.86	<<0.0001 significant
A	410.88	410.88	131.18	0.0001
B	45.80	45.80	14.62	0.0021
C	11.88	11.88	3.79	0.007
Lack of Fit	40.05	3.64	10.92	0.0868 not significant

P<0.05 indicates the model terms are significant while P<0.01 indicates the model term is highly significant

Table 4.7b Analysis of variance (ANOVA) for response surface quadratic model for antioxidant activity.

Source	Sum of squares	Mean square	F value	P>F
Model	3030.81	336.76	9.40	0.0005 significant
A	18.77	18.77	0.52	0.0001
B	0.40	0.40	0.011	0.0058
C	395.64	395.64	11.05	0.0008
AB	427.78	427.78	11.94	0.0106
AC	72.60	72.60	2.03	0.1975
BC	6.66	6.66	0.19	0.0079
A^2	631.41	631.41	17.63	0.0040
B^2	747.34	747.34	20.86	0.0026
C^2	951.85	951.85	26.57	0.0013
Lack of Fit	20.21	4.04	12.12	0.0779 not significant

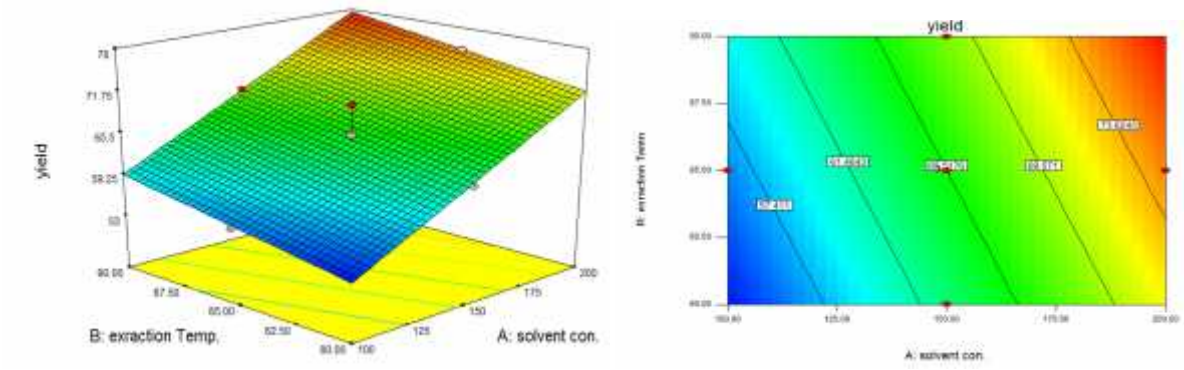
P<0.05 indicates the model terms are significant while P<0.01 indicates the model term is highly significant

4.5. Optimization of dependent variables or responses

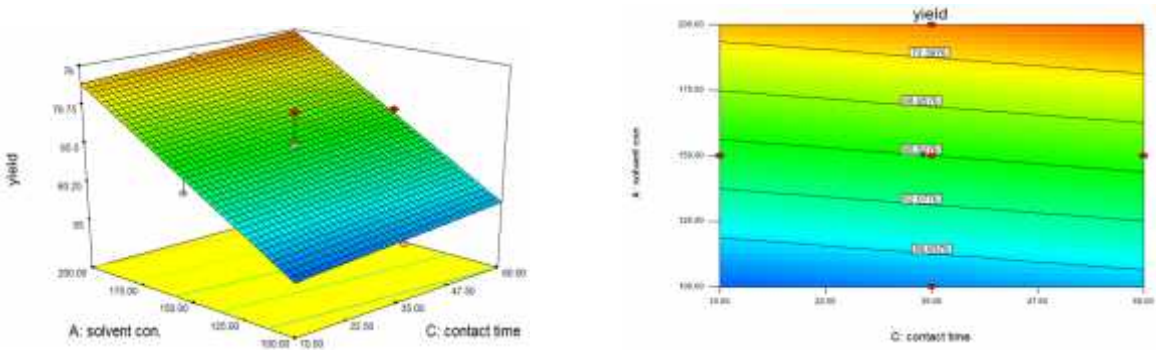
The relationship between independent and dependent variables was graphically represented by 3D response surface and 2D contour plots generated by the model (Figures 4.3-4.5). Different shapes of the contour plots indicated different interactions between the variables, an elliptical contour plot indicated the interactions between the variables were significant while a circular contour plot means otherwise.

Figure 4.3b showed the interaction between distilled water concentration (A) and extraction

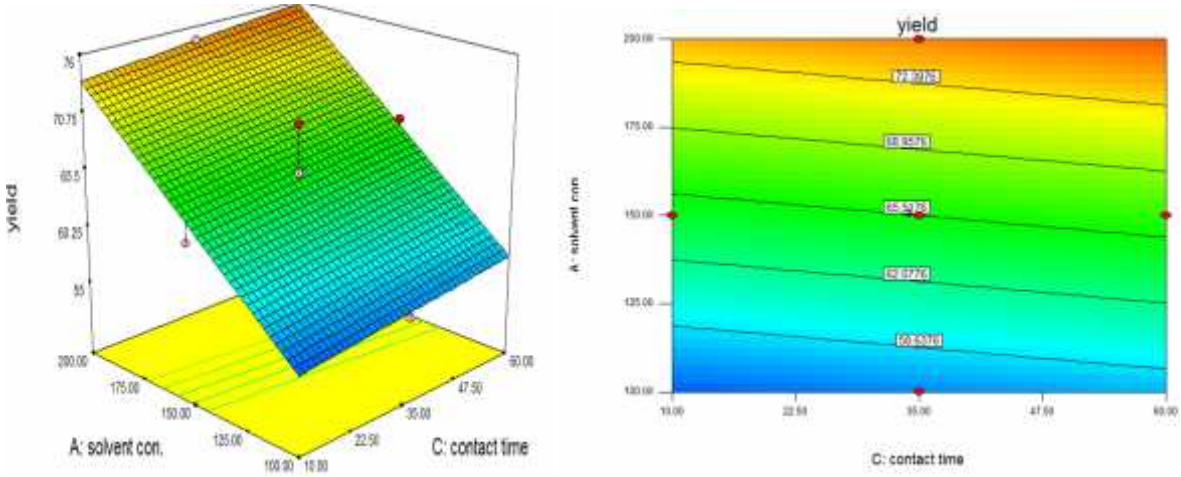
time (C) on the yield of green tea extracts. Increase of distilled water concentration from 100 to 200 ml and extraction time from 10 to 35 min improve the extraction yield. However, when the extraction time over 35min, did not show any obvious effect on extraction yield. The possible explanation could be that, increasing extraction time may accelerate chemical degradation of bioactive compound in extraction process, which resulted in the lower extraction yield. Figure 4.3a described the effect of distilled water extraction (A) and extraction temperature (B) on the yield of green tea extracts. It may be also observed that when extraction time(C) was fixed at 0 levels, extraction temperature (B) displayed a linear effect on the response yield. Varying distilled water concentration from 100 to 200ml with the increase of extraction temperature from 80 to 90℃, the target compounds' extraction yield was increasing up to 85℃ with the increase of distilled water concentration, but extraction temperature beyond 85℃ did not show any obvious effect on extraction yield. As shown in Figure 4.3c and Table 4.2, extraction solvent concentration is the main extraction parameters that positively affect the yield, but extraction temperature and extraction time had insignificant effect on the yield. But, it doesn't mean that totally haven't effect on the yield rather have a certain effect on the optimization process. The highest extraction yield was possibly achieved when using 80℃ of extraction temperature and 10min of extraction time.



(a) Response surface plot and contour plot of distilled water concentration and extraction temperature



(b) Response surface plot and contour plot of distilled water concentration and extraction time.

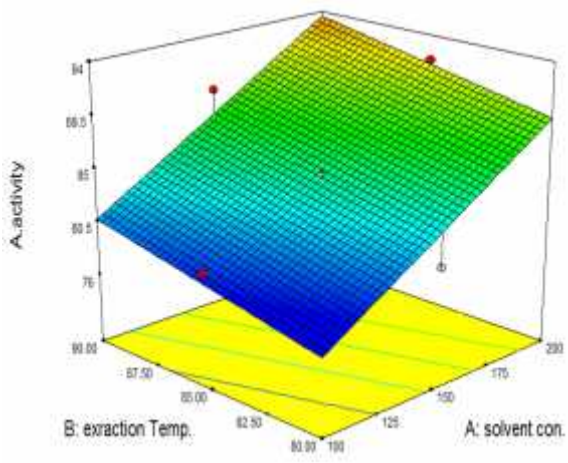


(c) Response surface plot and contour plot of distilled water concentration and extraction time.

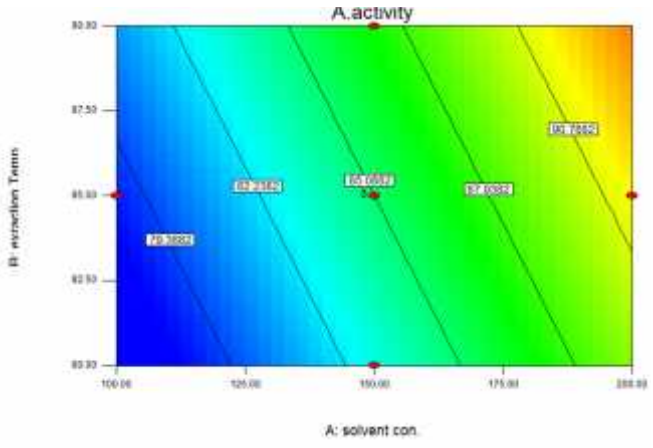
Figure 4.5 3D response surface and 2D contour plot showing the effect of extraction parameters on yield of green tea extracts.

On the other hand Figure 4.6 and Table 4.6 showed the interaction between distilled water concentration (A) and extraction time (B) have a significant effect on the antioxidant activity of green tea extracts. Increase of distilled water concentration from 100 to 200 ml and extraction temperature from 80 to 90 ℃ improves the extraction of antioxidant activity. But, both interaction effect of extraction solvent concentration (A) and extraction time (B) and that of extraction temperature and extraction time had no a significant effect on antioxidant activity, as shown in Figure 4.5b. However, the individual variable, extraction time had shown a significant effect on the

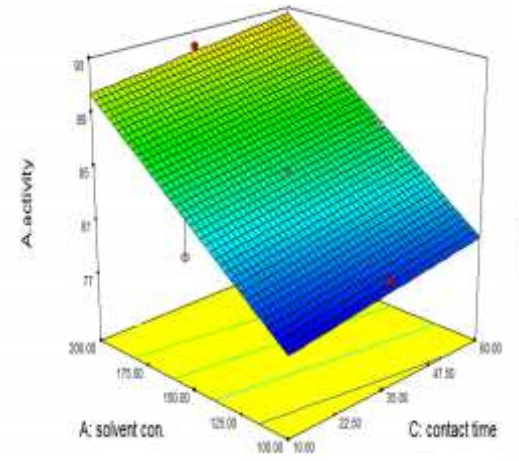
antioxidant activity of the extracts.



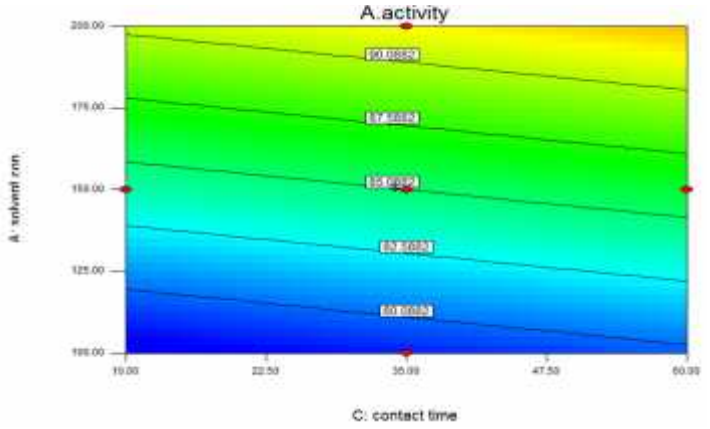
(a) Response surface plot of distilled Water and extraction temperature



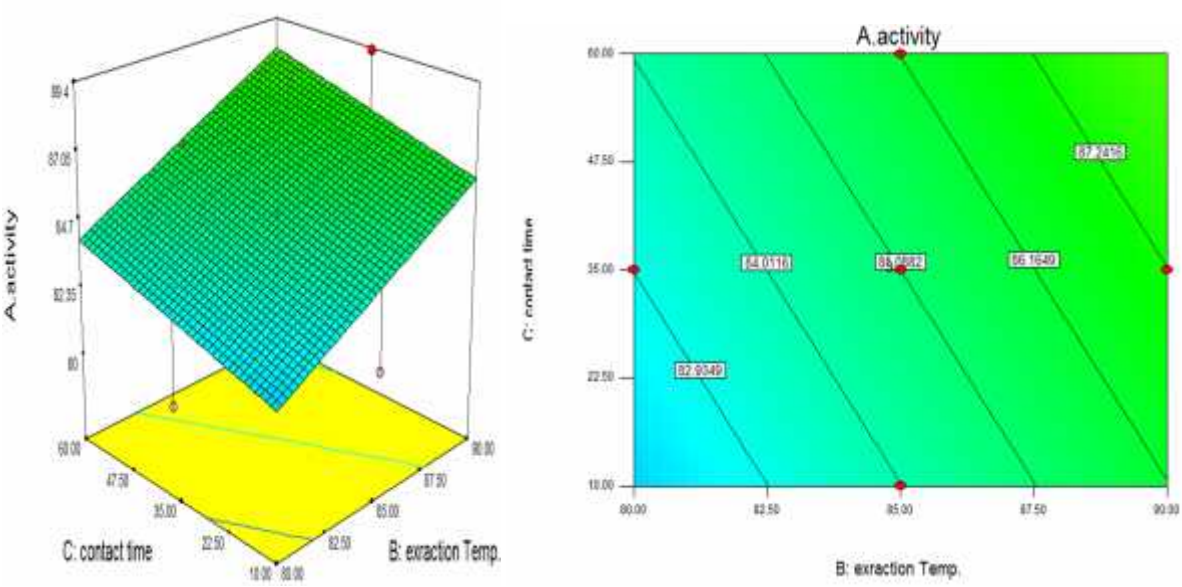
(b) Contour plot of distilled water and extraction temperature



(c) Response surface plot of distilled Water and extraction time



(d) Contour plot of distilled water and extraction time



(e) Response surface plot of extraction temperature and extraction time

(f) Contour plot of extraction temperature and extraction time

Figure 4.6. 3D response surface and 2D contour plot showing the effect of extraction parameters on antioxidant activity of green tea extracts.

4.5.1. Verification of predictive model

In this study, the aim of optimization was to find the conditions which gave the maximum extraction yield and antioxidant activity of green tea extracts. The software generated the optimum distilled water concentration, extraction temperature and extraction time was 200ml, 90 ℃ and 60 min, respectively. The software predicted the extraction yield and antioxidant activity of the extracts was **78.9076 %** and **96.8657%** with the desirability of 0.994, respectively. As shown in Table 4.6, two parallel experiments were carried out under the optimal conditions. Comparing with the predicted value by Design Expert version 7.0.0, the results showed that the actual value of yield and antioxidant activity was **79.8500%** and **96.9810%** which was very close to the actual results. This indicated that the optimization achieved in the present study was reliable.

Table 4.8. Optimum conditions and the predicted and experimental value of response at the optimum conditions.

	Distilled Water (ml)	Extraction temperature (℃)	Contact time (min)	Yield (%)	Antioxidant activity (%)
Optimum conditions (predicted)	200.0	90.0	59.99	78.9076	95.8657
Modified Conditions (actual)	200.0	88.0	35.0	79.8500	96.9810± 0.01
Error				0.9424	1.1153

4.6. Preservative effect of green tea extracts on Soybean and Niger seed oils

The preservative effect of green tea extracts was studied on refined Soybean and Niger seed edible oils. The potential preservative effect of the extracts was evaluated based on the chemical analysis of rancidity parameters. These rancidity parameters are Free Fatty Acids and Peroxide Value which indicates the deterioration of the model foods, edible oils, for this case.

The extracts concentrations that used for the preservative effect was 60µl which had high antioxidant activity as it was selected in section 4.3 of this manuscript. But, since this concentration is low and natural antioxidants have no any toxicity, the concentration levels were held as 0 (control treatment), 1ml and 2ml of extracts. The rancidity parameters, FFA and PV, of the refined soybean and Niger seed oils were measured for three consecutive weeks as per the storage duration of the oils.

Finally, the preservative effect of the extracts on the refined soybean and Niger seed oils were compared based on the value of FFA and PV and deduction had been given.

4.6.1. Free Fatty Acids (FFA)

Table 4.7a shows the results of FFA for both refined soybean and Niger seed oils treated with 0, 1and 2 ml of green tea extracts. This rancidity parameter of foods was determined using titration method for three consecutive weeks as per the storage duration of the oils sample. The results were

expressed by the percentage of oleic acid value.

As it is observed from Table 4.8a, the FFA of refined Niger seed oil was increased as the storage periods increased. But, FFA value of the refined Niger seed oil was decreased within the given storage week as the extracts concentrations amount increased. The FFA of refined Niger seed oil which was treated without the green tea extracts, control treatment, found to be 0.254±0.02, 0.382±0.01 and 0.46±0.02 mgKOH/g for the 1st, 2nd and 3rd weeks of storage time, respectively. Refined Niger seed oil treated with 1ml has FFA value of 0.224± 0.04and 0.362± 0.002 mgKOH/g at the 1st and 3rd weeks of storage time analysis, respectively. Whereas, refined Niger seed oil treated with 2ml has FFA value of 0.183± 0.01and 0.234±0.04 mgKOH/g at the 1st and 3rd weeks of storage time analysis respectively.

Table 4.9a FFA (mg KOH/g) of refined Niger seed oil treated with green tea extracts.

		Concentrations of green tea extracts (ml)		
Oil name	Storage/weeks	0	1	2
Refined Niger seed oil	1st	0.254±0.02	0.224± 0.04	0.183± 0.01
	2nd	0.382±0.01	0.281± 0.02	0.245 ±0.03
	3rd	0.460±0.02	0.362± 0.002	0.234±0.04

On the other hand, Table 4.8b showed the results of FFA values for refined soybean oil. As it is clearly seen from this table, the FFA values of refined oil were increased like that of refined Niger seed oil as the storage periods increased. The FFA values of refined soybean oil which was treated without the green tea extracts, control treatment, found to be 0.304±0.01, 0.482±0.01and 0.576±0.00 mgKOH/g for the 1st, 2nd and 3rd weeks of storage time, respectively. Refined soybean oil treated with 1ml has FFA value of 0.284± 0.04and 0.422± 0.002 mgKOH/g at the 1st and 3rd weeks of storage time analysis, respectively. Whereas, refined soybean oil treated with 2ml has FFA value of 0.203± 0.01 and 0.300±0.04 mgKOH/g at the 1st and 3rd weeks of storage time analysis respectively.

Table 4.9b FFA (mg KOH/g) of refined soybean oil treated with green tea extracts.

Oil name	storage/weeks	concentrations of green tea extracts (ml)		
		0	1	2
Refined soybean oil	1st	0.304±0.01	0.284± 0.04	0.203± 0.01
	2nd	0.482±0.01	0.341± 0.02	0.245 ±0.03
	3rd	0.576±0.00	0.422± 0.002	0.300±0.04

Alternatively, Figure 4.4 explained briefly the interaction of green tea extracts concentrations and Free Fatty Acids (FFA) of both refined Niger seed and soybean oils during the 3rd week storage time.

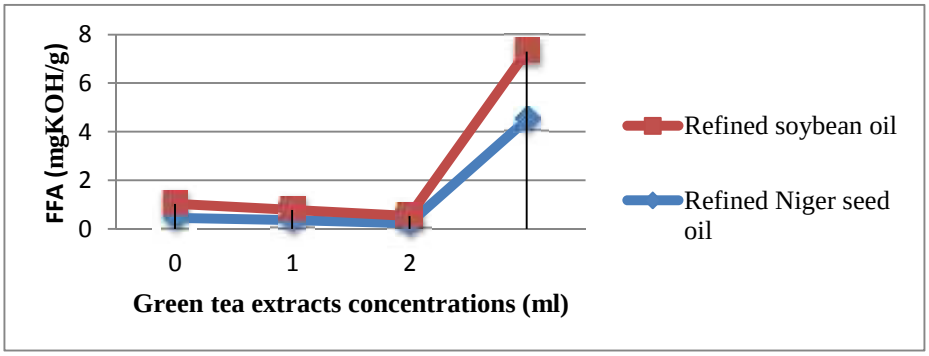


Figure 4.7 graphical representation of FFA value for refined Niger seed and Soybean oils treated with different concentration of green tea extracts during the 3rd week of storage time.

As it is observed from Table 4.8a, Table4.8b and Figure 4.6 FFA values of both refined Niger seed and soybean oils were increased as the storage time increased for both control and extracts treated. Whereas, FFA values of refined Niger seed and soybean oils were decreased within each weeks of storage periods. FFA values of both oils decreased in the order of 0>1>2 ml concentrations of the extracts. Both refined Niger seed and soybean oils treated with green tea extracts had less FFA values than those oils treated with no green tea extracts, control treatment. This showed that green tea extracts significantly reduce oxidative rancidity occurred in the oils. Because as (Golding *et al.*, 2011) reported that catechins of green tea extracts have high potency for preventing lipid

peroxidation of oil-containing foods; they scavenge free radicals and stop the auto-oxidative degradation of lipids. Similarly, (Golding *et al.*, 2011) reported that catechins, especially epigallocatechin gallate (EGCG), have been found to have more than 20 times the relative potency of vitamin E for preventing lipid peroxidation and more than 4 times the relative potency of butylated hydroxyanisole (BHA), which is often added in food to preserve fat or oil.

Indeed, green tea extracts potentially preserve both refined Niger seed and soybean oils. But, the potency of preservation was depended on its concentration as it was observed in both the above tables. The FFA values of the selected food model had acceptable value which is agree with the AOCS, that is the acceptable levels for all oil samples should be below 0.6 mg KOH/g (measured in potassium hydroxide per gram) (AOCS Official Method Cd 8-53, 2003). Thus, the research found out the potential preservative effect of green tea extracts on refined Niger seed and soybean oils.

4.6.2. Peroxide Value (PV)

According to Table 4.9, per oxide value of both refined Niger and soybean oils were varied with storage times. PV is a measure of oxidation during storage and the freshness of lipid matrix. In addition, it is a useful indicator of early stages of rancidity occurring under mild condition and it is a measure of the primary lipid oxidation products. One of the most important parameters that influence lipid oxidation is the degree of unsaturated fats are oxidized; peroxides are among the oxidation products formed. High peroxide value is an indicator of oxidation level and the greater the peroxide value, the more oxidized the oil is (http://**www**.bioriginal.com).

As it was seen from Table 4.8a and Table 4.8b, the PV of refined Niger seed and soybean oils treated with no green tea extracts , control treatment, were 4.80±0.12 and 12.30±0.01 and 4.20±0.00 and 16.50±0.15 meq O_2 /kg of the samples for the 1st and 3rd weeks analysis, respectively. Whereas, PV of refined Niger seed oil treated with 1ml of green tea extracts was 4.0± 0.30 and 9.70±0.21 meq O_2/kg of oil samples for the 1st and 3rd weeks analysis, respectively and while with 2ml extracts was 3.50± 0.5 and 7.41±0.05 meq O_2 /kg of oil samples for the 1st and 3rd weeks analysis, respectively.

On the other hand, PV of refined soybean oil treated with 1ml of green tea extracts was 3.80± 0.04 and 12.1± 0.15 meq O_2 /kg of oil samples for the 1st and 3rd weeks analysis, respectively whereas, with 2ml of green tea extracts was 3.00±0.02 and 9.00±0.05 meq O_2 /kg of oil samples for the 1st and 3rd weeks analysis, respectively.

Table 4.10a PV (meq O_2/kg)of refined Niger seed oil treated with green tea extracts.

		Green tea extracts concentrations (ml)		
		0	1	2
Oil name	storage/weeks			
Refined Niger seed oil	1st	4.80±0.12	4.0±0.3	3.50± 0.5
	2nd	8.20±0.10	7.3±0.2	5.8±0.05
	3rd	12.30±0.01	9.7±0.21	7.41±0.05

Table 4.1b PV (meq O_2/kg)of refined soybean oil treated with green tea extracts.

		Green tea extracts concentrations (ml)		
Oil name	storage/weeks	0	1	2
Refined soybean oil	1st	4.20±0.01	3.80 ±0.04	3.00±0.02
	2nd	9.50 ± 0.03	7.50±0.03	6.20±0.02
	3rd	16.50±0.15	12.10±0.02	9.00±0.05

Most peroxide values of both oil samples were in agreement with the maximum codex standard peroxide value (10meq O_2 /kg) for vegetable oil deterioration. Both oil samples treated with no green tea extracts have significantly high peroxide values at the 3rd week of storage time. Hence, refined Niger seed and soybean oils treated with no green tea extracts have a PV value of 12.30±0.01 and 16.50±0.15 meq O_2 /kg respectively, at the 3rd week of storage time. Whereas both oil samples treated with 1 and 2 ml of green tea extracts have relatively less PV at the 3rd week of storage time. Accordingly, both refined Niger seed and soybean oils treated with 1ml of green tea extracts have a PV of 9.7±0.21 and 12.1±0.02 meq O_2 /kg respectively, at the 3rd weeks of storage time. Whereas oil samples of Niger seed and soybean oils treated with 2ml of green tea extracts have PV of 7.41±0.05 and 9.00±0.05 meq O_2 /kg respectively, at the 3rd week of storage time. This

implies that refined soybean oil has high degree of unsaturation and suggested that it contains high amount of unsaturated fatty acids, linoleic (C18:2) and oleic (C 18:1), which are responsible for oxidative rancidity (Belete and Dimberu, 2011).

This study indicates that both oil samples having higher Pv_s, which is very likely in products that do not contain preservatives, either natural or synthetic, rather has naturally occurred preservative, vitamin E. This study also reveals that green tea extracts has a potential preservative effect on fat based foods.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATION

5.1. Conclusions

Non-fermented green tea powders have essential compounds like polyphenols which imparts green tea having excellent antioxidant potential. The aim of this study was to extract these compounds and evaluate its antioxidant and preservative potential on refined non preservative Niger seed and soybean oils.

Extraction of antioxidants was conducted by varying extraction parameters, namely extraction solvent (distilled water), extraction temperature and contact time. Extraction was performed at solvent concentration (100, 150 and 200ml), extraction temperature (80, 85 and 90℃) and contact time (10, 35 and 60min) with 10g of powder green tea. High extraction yield (78.3%) and antioxidant activity (96.1%) was obtained at 200ml, 90℃ and 60min. On the other hand optimization of the responses, yield and antioxidant activity, making the extraction parameters in range were performed by Design Expert version 7.0.0 software. Accordingly, the optimal predicted yield and antioxidant activity were 78.807% and 94.7277% at 90 ℃ and 59.99min, respectively. Whereas the actual optimal values were 79.850% and 96.9810 ±0.01 % at modified conditions, 88.0℃ and 35.0min respectively.

The antioxidant activity of green tea extracts was compared with standard Ascorbic acid by varying the concentration of the extracts by free radical scavenging activity, DPPH, using UV-spectrophotometer. The concentrations of the extracts and standard Ascorbic acid were 20, 40, 60, 80, 100 and 120µl. As this study revealed that the antioxidant activity of green tea extracts increased as the concentrations increased, but almost constant after 60µl of the extracts, as shown on Table 4.3 of this manuscript. Whereas the standards antioxidant's was constant in all concentrations. As Figure 4.1 of section 4 showed that at the beginning concentrations of the extracts the antioxidant activity of green tea was slightly differ from the standard ascorbic acid but overlap/ equivalent after 40 µl of the extracts.

On the other hand, the preservative potential of green tea extracts was evaluated on refined non

Niger seed and Soybean oils. The preservative effect of the extracts was evaluated based on the chemical analysis as per the storage times. Free Fatty Acids (FFA) and Peroxide Values (PV) of both samples without green tea extracts (control) were increased during the three weeks storage. As the result showed, FFA and PV of both oil samples treated with 1 and 2ml of green tea extracts highly decreased these oxidation quality parameters.

In conclusion, this study used as an eye breaker for food and medicinal sectors who are interested to engage in manufacturing natural antioxidant from non-fermented green tea in addition to manufacturing tea as a beverage food. Based on the result, incorporating 1 and 2ml of green tea extracts on fat and oil based food products and others significantly improves the quality of foods and retards the oxidative rancidity. Hence, non-fermented green tea has excellent antioxidant activity and preservative potential as observed on refined non preservative Niger seed and soybean oils.

5.2. Recommendation

Extracts of green tea powder might have essential compounds rather than polyphenols which promote potential antioxidant. Thus, study should be conducted on the isolation, composition and quantification of green tea powder compounds by sophisticated equipment such as High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC).

Comparative study should be conducted on the extraction and isolation of antioxidant from green tea powder using different organic solvents like Ethanol , Methanol , Hexane, petroleum ether and others with equipment like Supercritical extractor, Microwave assisted extractor and others. Antioxidant activity of natural materials can be determined using different techniques rather than DPPH free radical scavenging activity like ferric reducing ability of plasma (FRAP) assay and Rancimat methods.

Farther, the preservative potential of green tea extracts can also be evaluated on different food products like butter, ghee, cheese, salad, and meat. In general this study gave the following promising research area for farther study.

- ✓ Production of green tea based cosmetics
- ✓ Antimicrobial potential of green tea extracts
- ✓ Isolation of green tea catechins
- ✓ Comparison of green tea and black tea antioxidant activity, antimicrobial effect and preservative effect on butter, oil, ghee and meat.

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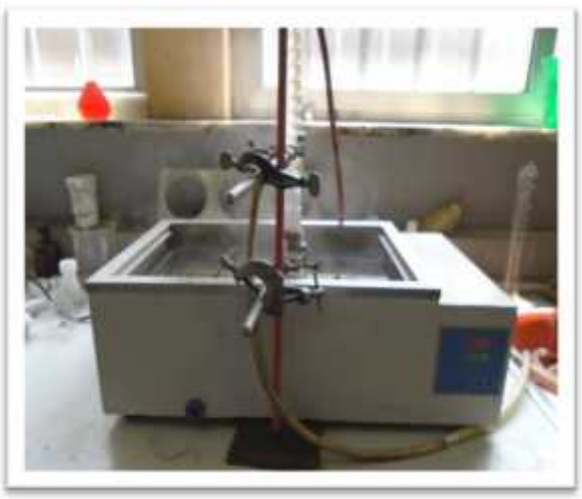
Appendices

Appendice A: Some important shooted photo during experimental session



(a) Water bath

(b) Crude green tea extraction process

(c) Vacuum filtration of the extracts

(d) Poly lab. Tubes for keeping filtrate

(e) Sample + DPPH solution (f) Blank sample (Methanolic DPPH only)

(g) Computerized UV-Spectrophotometer (h) Testing antioxidant potential of green tea Crude extracts

Appendice B: Raw data for antioxidant activity evaluation by UV-Spectrophotometer at 517nm

Run	Extracts con. (µL)	Absorbance at 517nm	Blank DPPH soln. (ml)	Absorbance at 517nm
1	S_1= 20	0.27900	B_1=4	0.53410
	S_2= 20	0. 35830	B_2=4	0.42500
	S_{av}=20	A_{sav}= 0. 31865	B_{av} =4	A_{bav}= 0.47955
	S_1= 40	0.16400	B_1 =4	0.53410
	S_2= 40	0.13070	B_2 =4	0.42500
	S_{av}=40	A_{sav} =0.14735	B_{av} =4	A_{bav} =0.47955
	S_1= 60	0.0433	B_1 =4	0.5341
	S_2= 60	0.0426	B_2 =4	0.4250
	S_{av}=60	A_{sav} = 0.04295	B_{av} =4	A_{bav} =0.47955
	S_1= 80	0.03930	B_1 =4	0.5341
	S_2= 80	0.03930	B_2=4	0.4250
	S_{av}=80	A_{sav} = 0.03930	B_{av} =4	A_{bav} = 0.47955
	S_1= 100	0.04060	B_1= 4	0.5341
	S_2= 100	0.03950	B_2= 4	0.4250
	S_{av}= 100	A_{sav} = 0.04005	B_{av}= 4	A_{bav} = 0.47955
	S_1= 120	0.03900	B_1= 4	0.5341
	S_2= 120	0.04190	B_2= 4	0.4250
	S_{av}=120	A_{sav} = 0.04045	B_{av}= 4	A_{bav} = 0.47955
2	S_1= 20	0.5327	B_1= 4	0.8074
	S_2= 20	0.6060	B_2= 4	0.8988
	S_{av}= 20	A_{sav}= 0.56935	B_{av}= 4	A_{bav}= 0.8531
	S_1= 40	0.3986	B_1= 4	0.8074
	S_2= 40	0.3559	B_2= 4	0.8988
	S_{av}= 40	A_{sav} =0.37725	B_{av}= 4	A_{bav} = 0.8531
	S_1= 60	0.1642	B_1= 4	0.8074
	S_2= 60	0.1731	B_2= 4	0.8988
	S_{av} =60	A_{sav} = 0.16815	B_{av} = 4	A_{bav} = 0.8531
	S_1= 80	0.0647	B_1= 4	0.8074
	S_2= 80	0.0621	B_2= 4	0.8988
	S_{av}= 80	A_{sav} = 0.0634	B_{av} = 4	A_{bav} = 0.8531
	S_1= 100	0.04060	B_1= 4	0.8074
	S_2= 100	0.03950	B_2= 4	0.8988
	S_{av}=100	A_{sav} = 0.04005	B_{av}= 4	A_{bav} = 0.8531

3				
	S_1= 120	0.03900	B_1= 4	0.8074
	S_2= 120	0.04190	B_2= 4	0.8988
	S_{av}= 120	A_{sav} =0.04045	B_{av}=4	A_{bav} =0.8531
	S_1= 20	0.47932	B_1= 4	0.9567
	S_2= 20	0. 5568	B_2= 4	0.9581
	S_{av}= 20	A_{sav}=0. 5150	B_{av}= 4	A_{bav}= 0.9574
	S_1= 40	0.2887	B_1= 4	0.9567
	S_2= 40	0.3220	B_2= 4	0.9581
	S_{av}= 40	A_{sav} =0.3053	B_{av}= 4	A_{bav} =0.9574
	S_1= 60	0.1469	B_1= 4	0.9567
	S_2= 60	0.0987	B_2= 4	0.9581
	S_{av} = 60	A_{sav} = 0.1228	B_{av}=4	A_{bav} = 0.9574
	S_1= 80	0.0549	B_1= 4	0.9567
	S_2= 80	0.0515	B_2= 4	0.9581
	S_{av} =80	A_{sav} = 0.0532	B_{av}= 4	A_{bav} = 0.9574
	S_1= 100	0.04060	B_1= 4	0.9567
	S_2= 100	0.03950	B_2= 4	0.9581
	S_{av}= 100	A_{sav} = 0.04005	B_{av}=4	A_{bav} = 0.9574
	S_1= 120	0.0600	B_1= 4	0.9567
	S_2= 120	0.0595	B_2= 4	0.9581
	S_{av}=120	A_{sav} =0.0597	B_{av}=4	A_{bav} = 0.9574
4	S_1= 20	0.2001	B_1= 4	0.3459
	S_2= 20	0. 0547	B_2= 4	0.1824
	S_{av}=20	A_{sav}= 0. 1274	B_{av}=4	A_{bav}= 0.2641
	S_1= 40	0.0491	B_1= 4	0.9567
	S_2= 40	0.0435	B_2= 4	0.9581
	S_{av}=40	A_{sav} =0.0463	B_{av}=4	A_{bav} = 0.9574
	S_1=60	0.0455	B_1=4	0.9567
	S_2=60	0.0415	B_2=4	0.9581
	S_{av}=60	A_{sav} =0.0435	B_{av}=4	A_{bav} =0.9574
	S_1= 80	0.0421	B_1=4	0.9567
	S_2= 80	0.0433	B_2=4	0.9581
	S_{av} =80	A_{sav} =0.0427	B_{av}=4	A_{bav} = 0.9574
	S_1=100	0.0417	B_1=4	0.9567
	S_2=100	0.0416	B_2=4	0.9581
	S_{av}=100	A_{sav} = 0.0416	B_{av}=4	A_{bav} = 0.9574
	S_1=120	0.0425	B_1=4	0.9567

	S_2= 120	0.0423	B_2=4	0.9581
	S_{av}=120	A_{sav} = 0.0424	B_{av}=4	A_{bav} = 0.9574
5	S_1= 20	0.9624	B_1=4	1.0090
	S_2= 20	0. 8133	B_2=4	1.0000
	S_{av}=20	A_{sav}= 0. 8878	B_{av}=4	A_{bav}=1.0045
	S_1= 40	0.5750	B_1=4	1.0090
	S_2= 40	0.5452	B_2=4	1.0000
	S_{av}=40	A_{sav} = 0.5601	B_{av}=4	A_{bav}=1.0045
	S_1= 60	0.3100	B_1=4	1.0090
	S_2= 60	0.3507	B_2=4	1.0000
	S_{av}=60	A_{sav} =0.3303	B_{av}=4	A_{bav} =1.0045
	S_1=80	0.0923	B_1= 4	1.0090
	S_2=80	0.0935	B_2= 4	1.0000
	S_{av}=80	A_{sav} = 0.0923	B_{av}= 4	A_{bav} =1.0045
	S_1=100	0.2106	B_1= 4	1.0090
	S_2=100	0.2054	B_2= 4	1.0000
	S_{av}=100	A_{sav} =0.2080	B_{av}=4	A_{bav} =1.0045
	S_1=120	0.1146	B_1= 4	1.0090
	S_2=120	0.1370	B_2= 4	1.0000
	S_{av}= 120	A_{av}=0.1258	B_{av}= 4	A_{av}=1.0045

NB: this raw data is only for the first five runs.

Appendice C: Predicted and Actual values of responses

Response 1: Yield of green tea extracts

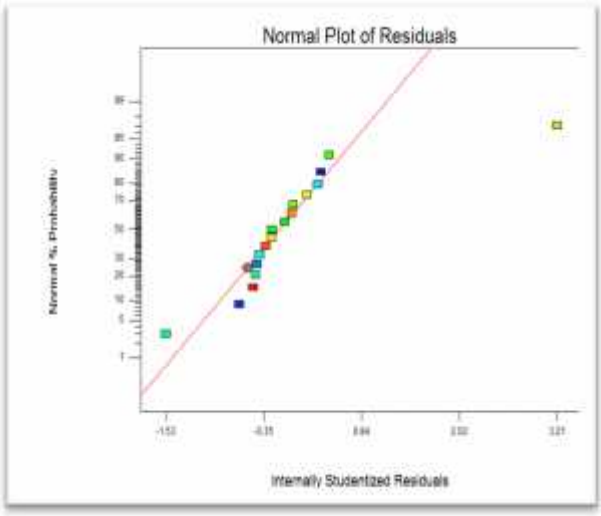
Standard order	Actual values	Predicted values	Residual
1	52.60	52.23	0.37
2	70.80	70.61	0.19
3	58.50	58.17	0.33
4	76.20	76.55	-0.35
5	53.80	54.49	-0.69
6	72.60	72.87	-0.27
7	60.00	60.43	-0.43
8	78.30	78.81	-0.51
9	55.80	56.33	-0.53
10	74.70	74.71	-0.01
11	62.00	62.55	-0.55
12	68.50	68.49	0.012
13	62.50	64.39	-1.89
14	67.20	66.65	0.55
15	69.70	65.52	4.18
16	65.40	65.52	-0.12
17	65.20	65.52	-0.32

Response 2: Antioxidant activity of crude green tea extracts

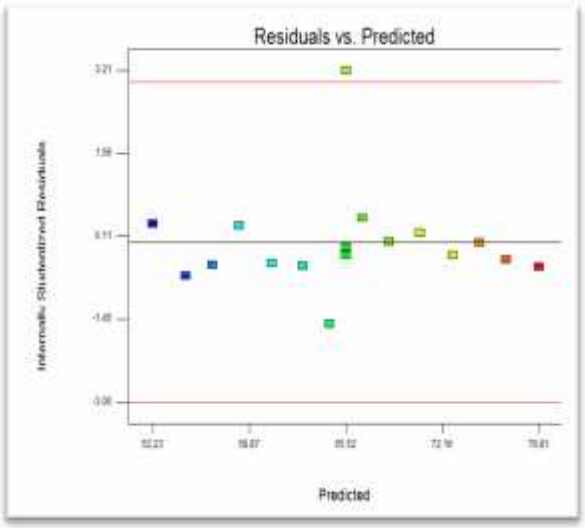
Standard order	Actual values	Predicted values	Residual
1	78.00	75.45	2.55
2	89.90	88.27	1.63
3	80.00	79.73	0.27
4	92.60	92.55	0.052
5	78.80	77.63	1.17
6	90.90	90.45	0.45
7	81.00	81.91	-0.91
8	96.10	94.73	1.37
9	79.50	78.68	0.82
10	91.90	91.50	0.40
11	80.00	82.95	-2.95
12	89.30	87.23	2.07
13	81.20	84.00	-2.80
14	85.80	86.18	-0.38
15	83.50	85.09	-1.59
16	83.50	85.09	-1.59
17	4.50	85.09	-0.59

Appendice D: Model adequacy checking graphs for responses

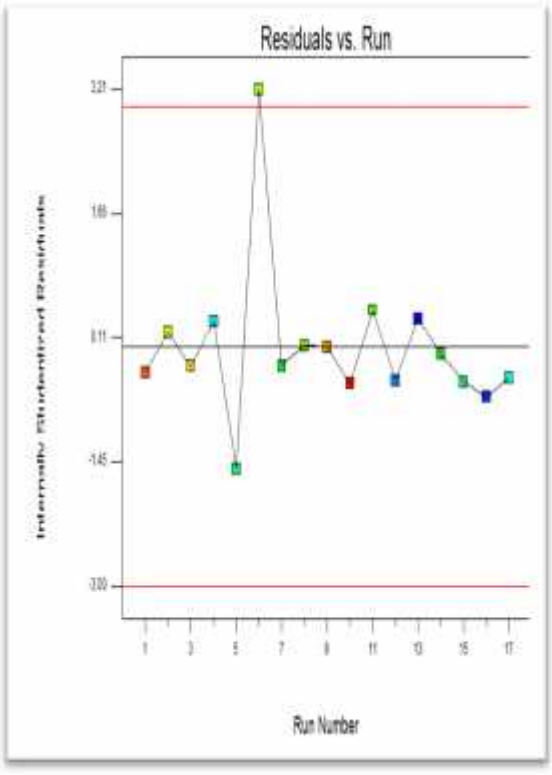
Response 1: For Yield



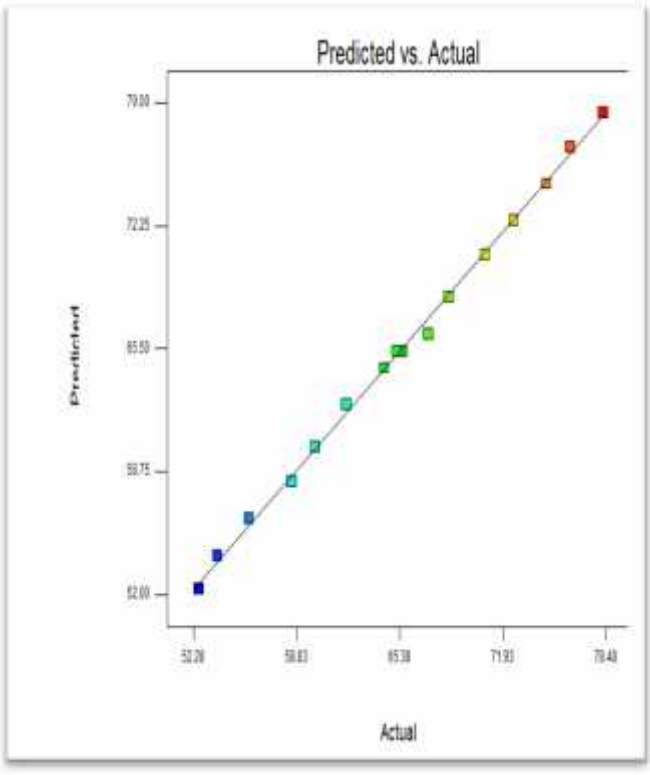
(a) Normal plot of Residuals



(b) Residuals versus Predicted

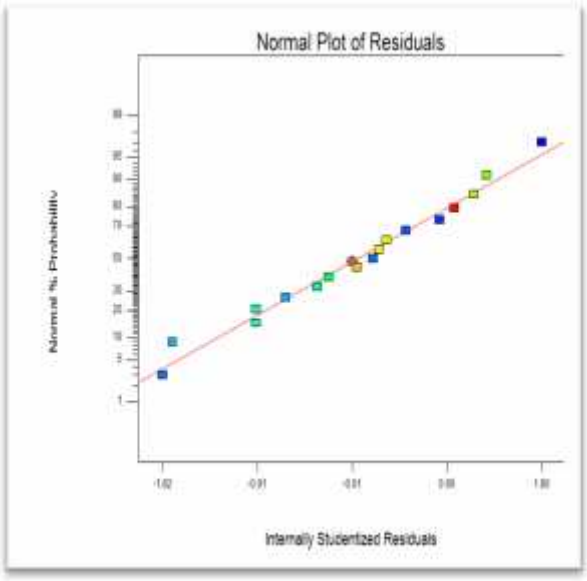


(c) Residuals versus Run

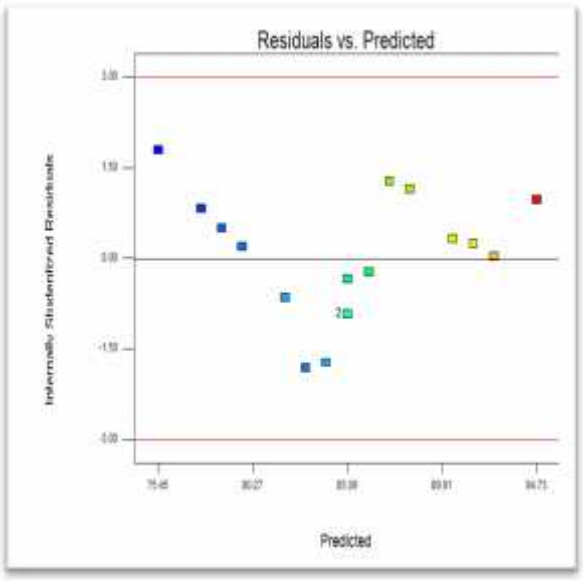


(d) Predicted versus Actual

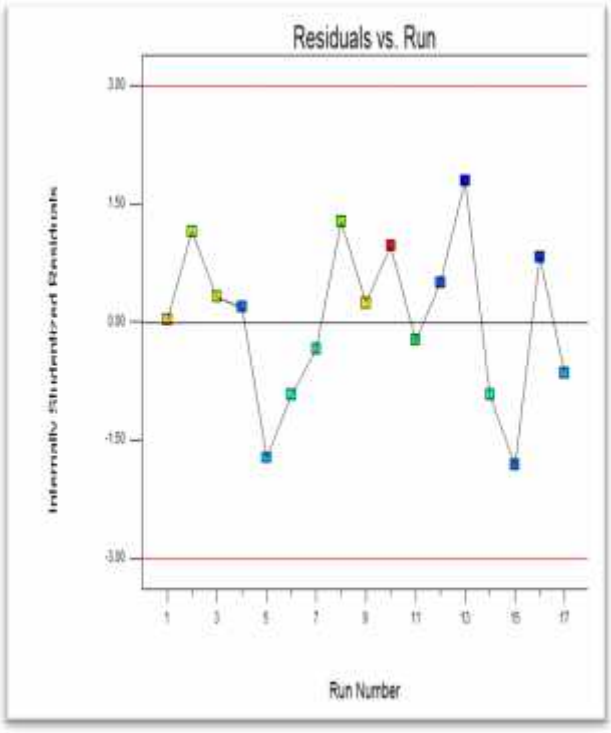
Response 2: For Antioxidant activity



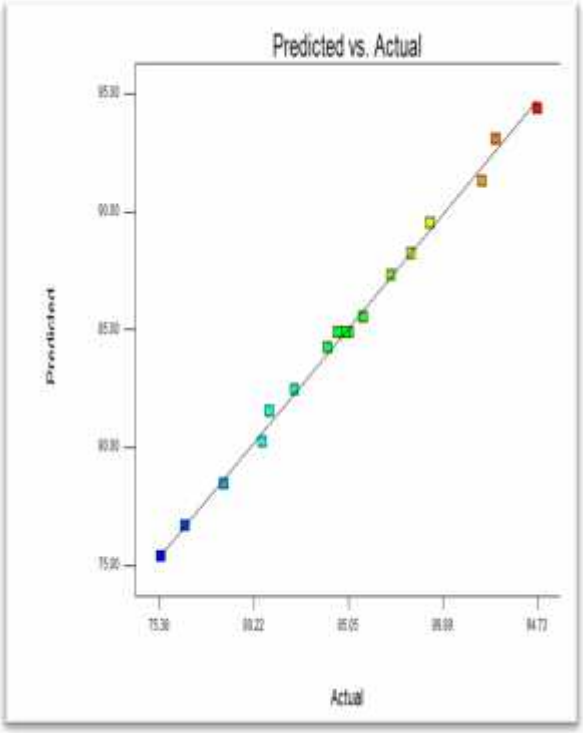
(a) Normal plot of Residuals



(b) Residuals versus Predicted



(c) Residuals versus Run



(d) Predicted versus Actual

Appendice E: Report of numerical optimization on antioxidant extraction process

Constraints Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Solvent con.	in range	100	200	1	1	3
Extraction Temperature	in range	80	90	1	1	3
Contact Time	in range	10	60	1	1	3
Yield	maximize	52.6	78.3	1	1	3
Antioxidant activity	maximize	78	96.1	1	1	3

Solutions

No	Solvent Con.	Extraction Temperature	Contact Time	Yield	Antioxidant Activity	Desirability	
1	200.00	90.00	60.00	78.7076	95.8657	0.994	Selected
2	200.00	89.96	60.0	78.8866	95.8445	0.993	
3	200.00	90.0	59.57	78.8844	95.8355	0.993	

DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any University and that all the sources of materials used for the thesis has been duly acknowledged.

Shimelis Shumi Raya _____

Signature

Place : Addis Ababa, Ethiopia

Date of submission : _____

This thesis has been submitted for examination with my approval as university advisor

Advisor Eng. Gizachew Shiferaw (Ass. Professor) _____

Signature