INVESTIGATION OF NATURAL AND PROCESSED COCOA AND COCOA CONTAINING PRODUCTS FOR THEIR ANTIOXIDANT CAPACITY AND PHENOLIC COMPOUNDS

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INVESTIGATION OF NATURAL AND PROCESSED COCOA AND COCOA CONTAINING PRODUCTS FOR THEIR ANTIOXIDANT CAPACITY AND PHENOLIC COMPOUNDS

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ABSTRACT

INVESTIGATION OF NATURAL AND PROCESSED COCOA AND COCOA CONTAINING PRODUCTS FOR THEIR ANTIOXIDANT CAPACITY AND PHENOLIC COMPOUNDS

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Cocoa is one of the richest sources of polyphenols which have positive effects on cardiovascular health. (-)-Epicatechin(EC) and (+)-Catechin(C) are flavanols with several biological activities, particularly known for their high antioxidant potential. They scavenge free radicals in human body and prevent lipid peroxidation, which otherwise damage the cells. Food processing techniques may reduce the polyphenol contents and antioxidant capacity of food products substantially. Therefore, in this study natural and processed (alkaline) cocoa samples obtained from industry were investigated for their antioxidant capacities using $DPPH^{\bullet}$ and $ABTS^{\bullet}$ radical scavenging methods. EC and C contents of the cocoa samples and cakes prepared by these samples were evaluated using RP-HPLC.

IC_{50} for $DPPH^{\bullet}$ radical scavenging activity for natural and alkalized cocoa samples were determined as 0.047±0.001 mg/mL, and 0.064±0.001 mg/mL, respectively. Trolox equivalence data for natural and alkali samples were determined as 188.51±4.19 µmol TE/g and 71.84±2.99 µmol TE/g, respectively. Both total phenol and total flavonoid content analysis have shown that natural cocoa has the highest phenolic and flavonoid contents with 4883.84±382.72 mg GAE/100g and 3155.30±120.70 mg QE/100g, respectively. The HPLC results indicated that natural cocoa has the highest EC content with 0.936±0.17 mg/g and the alkalized cocoa has the highest C content 0.327±0.18 mg/g.

Natural cocoa and its products have much greater antioxidant activity, phenolic and flavonoid contents than alkali cocoa and their products. Further studies are necessary to improve the properties of natural cocoa and formalize polyphenol rich functional foods with significant health claims.

Keywords : Polyphenols, cocoa, (-)-Epicatechin, (+)-Catechin, HPLC

ÖZ

NATÜREL VE İŞLENMİŞ KAKAO VE KAKAO İÇERİKLİ GIDALARIN ANTİOKSİDAN KAPASİTESİ VE FENOLİK MADDELERİ YÖNÜNDEN ARAŞTIRILMASI

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Kakao, kardiyovasküler sistem üzerinde pozitif etkileri olduğu belirtilen en zengin polifenol kaynaklarından biridir. Epikatekin (EC) ve (+)-Katekin (C), çeşitlik biyolojik aktiviteleri olan, özellikle yüksek antioksidan potansiyelleri ile bilinen flavanollerdir. İnsan vücudunda oluşan serbest radikalleri yakalayabilirler ve hücrelere zarar verebilecek lipid peroksidasyonunu engelleyebilirler. Gıda işleme yöntemleri, gıda ürünlerinin polifenol içeriğini ve antioksidan kapasitelerini önemli ölçüde azaltabilmektedir. Bu nedenle bu araştırmada, endüstriden temin edilmiş olan doğal ve işlenmiş (alkali) kakao örneklerinin antioksidan kapasiteleri, $DPPH^{\bullet}$ ve $ABTS^{\bullet}$ radikali yakalama kapasiteleri ölçülerek belirlenmiştir. Kakao örneklerinin ve bu örnekler ile hazırlanan keklerin EC ve C miktarları, ters fazlı HPLC yöntemi ile tayin edilmiştir.

Natural ve işlenmiş alkali kakao örneklerinin DPPH radikali yakalama kapasitesi için IC_{50} değerleri, sırası ile 0.047±0.001 mg/mL, ve 0.064±0.001 mg/mL olarak belirlenmiştir. Natural ve işlenmiş alkali kakao örneklerinin Trolox dengi değerleri sırası ile 188.51±4.19 µmol TE/g and 71.84±2.99 µmol TE/g olarak belirlenmiştir. 4883.84±382.72 mg GAE/100g ve 3155.30±120.70 mg QE/100g ile natural kakaonun toplam fenolik ve flavonoid içeriklerinin her ikisinde de işlenmiş kakaoya göre daha yüksek olduğu belirlenmiştir. HPLC sonuçları, natural kakaonun 0.936±0.17 mg/g olarak EC miktarının, işlenmiş kakaonun ise 0.327±0.18 mg/g kakao olarak C miktarının daha yüksek olduğunu göstermiştir.

Natural kakao ve kakao ürünlerinin antioksidan aktivitesi, fenolik ve flavonoid içeriği, işlenmiş alkali kakao ve kakao ürünlerine kıyasla çok daha yüksektir. Natural kakaonun özelliklerinin iyileştirilmesi ve önemli sağlık etkileri olan polifenol içeriği zengin fonksiyonel gıdaların formülasyonu için ileri araştırmalara ihtiyaç vardır.

Anahtar Kelimeler : Polifenoller, kakao, (-)-Epikatekin, (+)-Katekin, HPLC

Dedicated to my family

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

FC: Folin-Ciocalteau

TPC: Total Phenol Content

GAE: Gallic Acid Equivalent

QE: Quercetin Equivalent

TE: Trolox Equivalent

NC: Natural Cocoa

AC:Alkalized Cocoa

NCC:Natural Cocoa Cake

ACC: Alkalized Cocoa Cake

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

DPPH: 2,2-diphenyl-1-picrylhydrazyl

TEAC: Trolox Equivalent Antioxidant Activity

RSA: Radical Scavenging Activity

IC_{50}: Half Maximal Inhibitory Concentration

HPLC: High Performance Liquid Chromatography

TFA: Trifluoroacetic Acid

THF: Tetrahydrofuran

EC:Epicatechin

C:Catechin

CHAPTER 1

INTRODUCTION

In recent years, the importance of nutrition was recognized and more people started to pay close attention to eating habits and food choices. The main reason for this behavior is that food habits affect their health and nutritionally balanced diets affect it positively. According to World Health Organization Statistics (WHO-Media center, n.d.) ischemic heart disease, stroke and chronic obstructive lung disease have remained the top major killer diseases during the past decade, and 7.4 million people died due to heart disease in 2012. Since this number is too high and it regularly increases, people and governments are interested in encouraging healthy diet and lifestyle. There are lots of risk factors that may cause heart disease such as; being overweight, high blood pressure, and an unbalanced diet high or lack of regular physical exercise (Whitney & Rolfes, 2007). However scientists focused on showing the association between nutrition and chronic diseases. The main reason for the top 3 causes of death, which are heart disease, cancer and stroke in USA (2006), is diet related. All this research indicates the importance of healthy foods. Especially research about the relation between the consumption of antioxidant rich foods and the risk of these diseases increased recently. Antioxidant rich foods have several beneficial effects on human health. However, the antioxidant effects of food sources depend on a number of different factors such as the type of the plant foods, freshness as well as processing variables. Although processed foods have the advantage of being available anytime and

anywhere more easily to reach than fresh foods, their antioxidant capacity and health effects differ significantly.

Cocoa is one of the most important plant derived antioxidant containing foods which is used in many different food products for daily consumption such as cakes, chocolates, biscuits, milk, pudding, etc. Because of its availability and high beneficial impact on health, cocoa and cocoa products attracted great attention in recent years. Therefore in this study, antioxidant and polyphenol analysis of natural and processed cocoa and cocoa products were investigated.

1.1 Cocoa

Cocoa has been known for many centuries are one of the most popular food stuffs and it is derived from cocoa bean plants. Cocoa plant has many different by products such as cocoa powder and cocoa butter, and different methods to obtain and produce cocoa have been present in the world for many years. According to those methods, cocoa can be alkalized, natural or defatted. It is possible to find many different products in different areas that contain cocoa, such as cakes with cocoa as a food stuff and crèmes containing cocoa butter as a pharmaceutical stuff. Moreover, cocoa is the main ingredient in chocolate, which is one of the most popular snacks in the world. Cocoa has a number of bioactive ingredients with reported clinical benefits on human health therefore there are a huge number of research studies carried out by food engineers, chemists, and clinicians in an interdisciplinary field to reveal the molecular mechanisms of cocoa and its bioactive components and their health benefits (Schinella et al., 2010; Corcuera et al., 2012). Even though it is widely used

and has known health-benefits, cocoa production and plantation is under threat in recent decades.

1.1.1 History of Cocoa

The Latin name *Theorem* means "food of the gods" and cacao is derived from the Aztec *xocolatl*, which is a combination of *xococ* (bitter) and *atl* (water). The history of cocoa is very old. According to Aztec and Mayan hieroglyphic writings, cocoa was in existence at least 2000 years ago. Since cocoa beans were so expensive and precious, their possession was an indicator of wealth and only wealthy people (royals, warriors) could eat chocolate. Therefore, it was also used in Mayan and Aztec ceremonies and religious events as a symbol of wealth. It was also used for different types and purposes, for example the hieroglyphs illustrate that Aztecs and Mayans drank cocoa powder suspended in water and used it as money in their shopping (History of Cocoa, n.d.). All of these historical findings indicate that cocoa is a very old food. Cocoa has retained its popularity for around 2000 years.

1.1.2 Cocoa Beans

The cocoa beans are the seeds of cacao tree (*Theobroma cacao* L.). The cacao tree (*Theobroma cacao* L.) grows in tropical regions, and its origin is in South and Central America (Beckett, 2008). Since the cacao tree likes hot, rainy climates, 20° north to 20° south of the equator are very suitable environments. There are 4 types of Theobroma cacao tree: Criollo, Forastero, Nacional and

Trinitario. Each type has different properties and cocoa quality. Cacao trees are delicate plants that like deep, rich, well-drained soil and grow naturally in the lower level of the evergreen rainforest; in this way, they are protected from wind, sun and environmental hazards. It is an evergreen with pinkish, white blossoms, and it begins to bear fruit at the age of three. The fruit are football-shaped pods and come in various colors. This pod contains sweet white pulp, juice and seeds. Each pod contains about 30-40 seeds, which are called cocoa beans (Afoakwa, 2010). Figure 1.1 presents the properties of cocoa beans (Martius, Eichler, & Urban, 1886 as cited in Theobroma cacao L., n.d,).

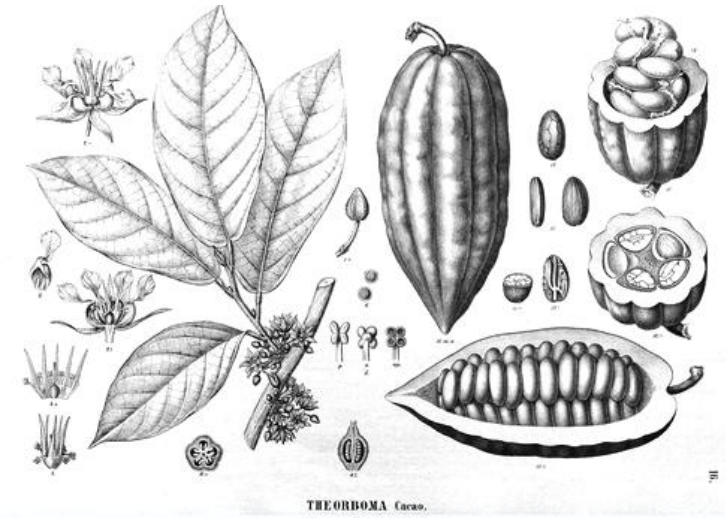


Figure 0-1 Picture of Cocoa plant, pod and cocoa beans

1.1.3 Cocoa Bean Processes

1.1.3.1 Fermentation

Cocoa bean processing starts with harvesting. After the pods are harvested, workers remove the beans and the pulp. When the beans removed from the pods, raw cocoa beans have a white color, and an unpleasant flavor and taste.

Therefore, they need to be fermented. The fermentation period may vary according to the type of bean but usually takes 2-3 days. The first stage of fermentation is stacking cocoa beans and covering them with banana leaves (Beckett, 2008). After the fermentation starts, the temperature increases quickly to 45 °C and this heat kills the beans. The sticky pulp undergoes ethanoic, acetic and lactic fermentations and during this process enzymes breakdown substances into smaller components (Beckett, 2008). The sugar and acid of the beans start to decompose, which shows that the chocolate flavor is forming. Fermentation has a crucial role in cocoa process because it gives specific flavors to the beans; for instance, one of the most important flavor-related effects is the reduction in the bitterness of the cocoa. On the other hand, this process causes a reduction in the polyphenol content of the cocoa beans. However, even though fermentation causes a known loss of polyphenols, without fermenting, achieving the special organoleptic properties of cocoa is not possible. After completing this fermentation process cocoa beans become ready for the next steps of processing and the next step is drying (Kim & Keeney, 1984).

1.1.3.2 Drying

Drying is essential in order to prevent any microbes and moulds from growing on beans. It also helps the development of its characteristic brown color. The length of the drying period depends on conditions; it may take longer or shorter. The fermented cocoa beans can be dried under the sun until 7-8% moisture level, which takes around one week. However, sun drying has contamination risks from the surroundings (Beckett, 2008). If the weather is wet and not sunny enough, cocoa beans can be dried by artificial drying such as wooden fires and forced air driers. The desired drying process takes place at a low temperature over a long time, which gives optimum taste and acidity to

the beans. After cocoa beans are fermented and dried, they are filled into the sacks and ready for storage or transport.

1.1.3.3 Cocoa Powder Production Process

Cocoa beans are exported all around the world from cocoa growing countries. During this transportation and storage period the moisture level of beans should be below 8%. Higher humidity will cause mold to grow. When the factory receives the beans, the first process is cleaning, during which process the impurities such as stone, iron, sand and plant material are removed. Then, the beans are roasted, which develops their special cocoa flavor. The roasting temperature and timing can vary from 90℃ to 170℃. During roasting, many physical and chemical changes take places, for example, loosening shells, darkening color, killing microorganisms, and loss of acidity and bitterness (Afaokwa, 2010). The bean roasting enables the shell to be easily removed from the nib. This shell removing process is called winnowing (Beckett, 2008) and the remaining part of cocoa bean is called the nib. After the winnowing process, the cocoa nibs are ready for grinding. The purposes of this grinding are to produce liquor with low viscosity and to obtain smooth cocoa powder. Normally the cocoa nib contains 55% cocoa butter and this cocoa butter is released into liquor by the grinding process. During this process heat treatment is applied in order to melt the cocoa nib and form chocolate liquor. At the end of the nib grinding process, the natural chocolate liquor is obtained.

The Alkalization process was first introduced by a Dutchman van Houten, in 1928 and is therefore named the Dutch process. Its aim is to increase the pH from 5.4 to 7, which causes color and flavor development in the liquor. Potassium or sodium carbonate alkali solutions are commonly used for this process (Afoakwa, 2010). Then the alkalized cocoa liquor is pressed by hydraulic presses in order to separate cocoa cake and cocoa butter. Generally

78–90% of the cocoa butter is collected by pressing and obtains high fat cake (22% fat) and low fat cake (10% fat). Finally, the cocoa cake is ground into smaller pieces and pulverized in order to obtain the desired type of cocoa powder. The following Figure 1-2 shows the process of cocoa powder production (Beckett, 2008).

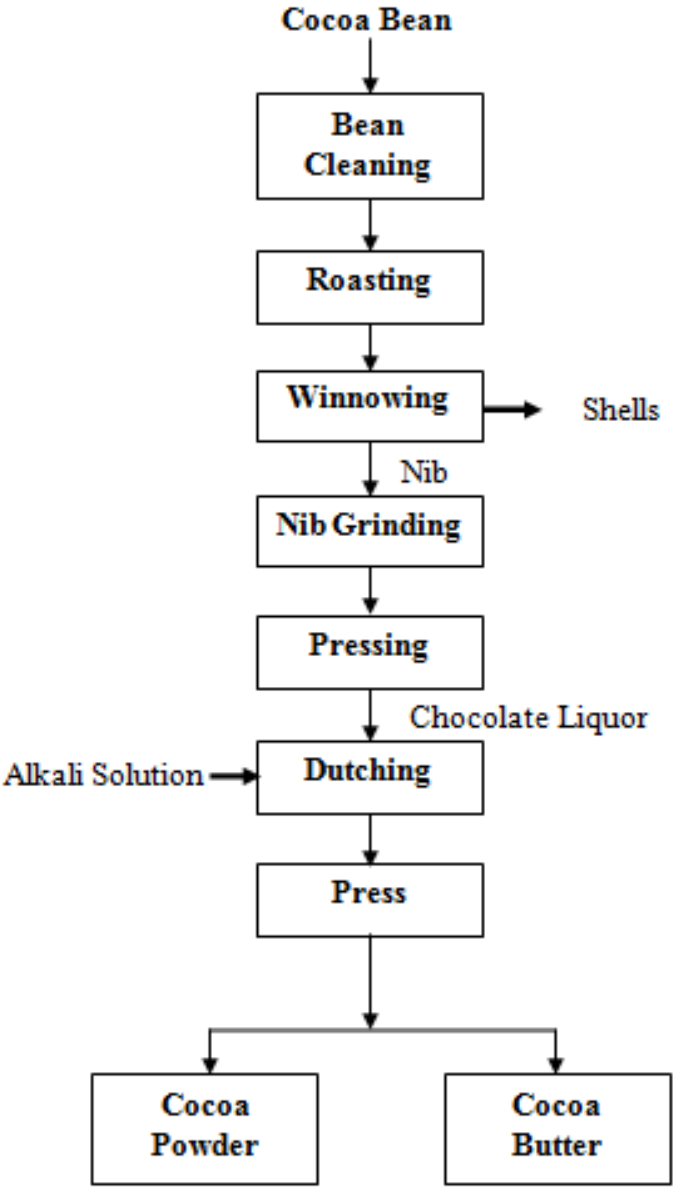


Figure 0-2 Flow chart of cocoa process

1.1.4 Nutritional data

The human body uses the organic nutrients carbohydrates, fat, and protein in order to provide energy. Each food has different nutritional facts, and similarly each person has different energy requirements. Generally, age, gender, body weight and height are the common factors that affect the energy requirements of a person. This means, each person has different energy and nutrient requirements. The average energy and nutrient requirements of a man and woman are indicated in Table 1-1.

Table 0-1 Nutrient Requirement of a Man and Woman

	Age (Years)	Ref BMI (kg/m)	Ref Height (cm)	Ref Weight (kg)	Energy (kcal/ day)	Carbohydrate (g/day)	Fat (g/day)	Protein (g/day)
Male	19-30	22.5	177	70	3067	130	18.6	56
Female	19-31	21.5	163	57	2403	130	13.1	46

*This table is adapted from Whitney and Rolfes (2007).

As it is observed from Table 1.1, a normal male, whose age is from 19-30, requires around 3000 kcal of energy and for a female in the same age range this amount is around 2500 kcal. However, people acquire this energy from different macronutrients and all different macronutrients have different contributions to the body in terms of energy. For example, 1 g of a protein and carbohydrate provides 4 kcal; on the other hand, 1 g of fat provides 9 kcal. It is important to get the required energy from all different macronutrients in order to provide a balanced diet. A balanced diet provides different DRI (Daily

recommended intake) for each macronutrient. This balanced diet protects the body against deficiencies of any nutrients such as minerals, proteins, vitamins, etc. According to DRI values, people should get 45-65% of their total daily required calories from carbohydrates, 20-35% from fat, and 10-35% from protein. These values are called Acceptable Macronutrient Distribution Ranges, or AMDR (Whitney & Rolfes, 2007)

The food pyramid illustrated in Figure 1.3 also shows the distribution of nutrients and indicates that the healthiest diet is the balanced diet because for all its different functions, the body consumes and requires different nutrients (Food pyramid, n.d.). For example, some vitamin D deficiencies in the body increase the risk of bone defects, or if a person does not consume any carbohydrates or fat, the body tries to get all its energy from protein and this causes the decreased usage of protein in the structural improvement in the body. Because of that, all these nutrients should be taken from different food sources. The food pyramid in Figure 1-3 shows how these nutrients can be provided by different food sources in a well-balanced diet.

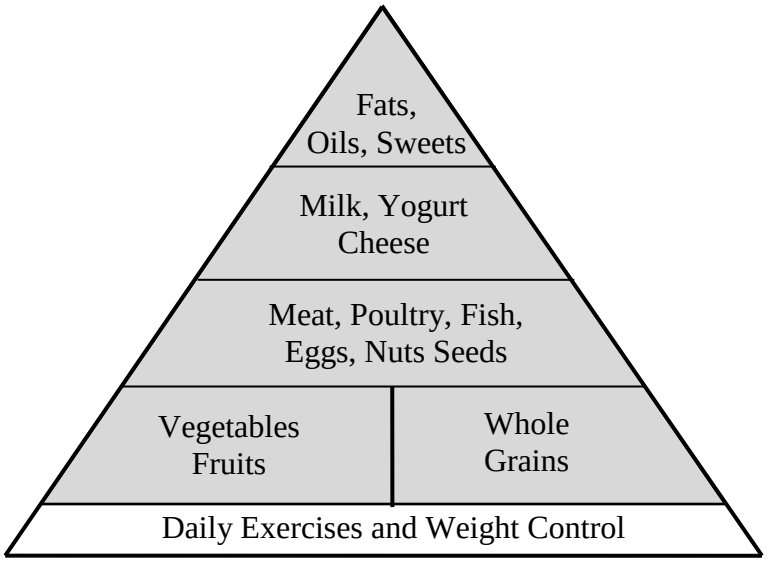


Figure 0-3 The food pyramid showing distribution of nutrition

According to this food pyramid, if someone relies on this multisource diet, it is possible to get all the RDI values of nutrients. In addition to all this information, the digestion of micronutrients such as minerals and vitamins are to be ingested and antioxidants, which are very important for a healthy body, can be obtained from a variety of food sources, as indicated in the food pyramid. There is different research indicating the required consumption rate of antioxidants in healthy bodies. Cocoa is the one of these beneficial antioxidant rich foods. Table 1-2 explains the nutritional values of cocoa products.

Cocoa beans show different physical and chemical properties according to origin and type of cocoa tree. Generally, cocoa beans contain a high amount of fat (more than 50%) named "cocoa butter" and rest is cocoa mass. Protein constitutes 10-15% and carbohydrates constitute 25-30 % of cocoa beans. Cocoa beans also contain "Theobromine", which has a similar effect to that of caffeine (Judelson, Preston, Miller, Munoz, Kellogg, & Lieberman, 2013).

Table 0-2 General solid composition of the cocoa powder (%)

	Cocoa Beans	**Cocoa Powder**
Carbohydrate	26.00	23.10
Fat	53.00	11.50
Protein	15.00	23.00
Fiber	11.00	26.90

During chocolate processing, cocoa butter and cocoa mass are obtained by the pressing of cocoa beans. By grinding cocoa mass, cocoa powder is obtained. The general composition of the cocoa powder is as follows (units/100 g): 23.0 g protein, 11.5 g fat, 23.1 g carbohydrate, 26.9 g fiber, 7.7 g minerals, 377 mg epicatechin, 135 mg catechin, 158 mg procyanidin B2, 96.1 mg procyanidin C1, 2192 mg theobromine, and 470 mg caffeine (Baba et al., 2007).

1.2 Oxidants and Antioxidants

In human life, antioxidants play quite an important role. Antioxidants are important for humans because of the multiple beneficial interactions they may be able to have within our bodies. Often this protection is dependent upon case, type, and location. For example, an antioxidant generated to help protect against lipid peroxidation in human tissues may or may not be able to prevent oxidative stress caused to DNA, proteins, or other compounds. There are three primary types of antioxidants found in nature. These include phytochemicals, vitamins, and enzymes (Hawaii, 2014). In nature, most powerful antioxidants are found in plants. In recent studies, there is increasing focus on natural foods providing antioxidants and food derived ones. They are mainly vitamins, phytochemicals and phenolic compounds. The main reason behind this increasing attention is related to the protective effect on oxidative damage in the body, which causes many diseases (Lee, Kim, Lee, & Lee, 2003)

Free radicals are molecules which has unpaired electrons. The main purpose of the antioxidant is the scavenging of free radicals, and the chelation of transition metals (Corcuera et al., 2012). Oxidation may be defined as electron transfer and as a result of this transfer, ATP is produced. However, when enough electrons cannot be provided to couple through this electron transfer, this causes free radical production. The role of antioxidants becomes important at this point. They provide a protective role against free radical formation or provide a reduction of oxidized molecules by scavenging unpaired electrons.

This free radical production is called oxidative stress in the body when it is increased. Oxidative stress is one of the main causes of serious diseases such as cancer, atherosclerosis, malaria, rheumatoid arthritis and even some brain defects resulting in memory disorders. With the help of antioxidants, free radicals increase or formation is prevented. The two main mechanisms behind the health benefits are:

- Any new free radicals are prevented from being produced. Example antioxidant enzymes: SOD (Superoxide dismutase) enzyme, catalase, glutathione reductases. $O_2\cdot$ is converted to H_2O_2 by SOD.
- Scavenging free radicals.

  Example antioxidants: Vitamin C, (-)-epicatechin, α-tocopherol, beta carotene. By scavenging a free radical, any chain oxidation reaction is prevented.

In addition different free radicals in the cells such as superoxide radical (O2•-) and hydroxyl radical (•OH) also present. Hydrogen peroxide is produced by this species and they may damage the cells in different parts of the body. The starting point of this damage is be expressed when they are initiating the chemical reactions such as lipid peroxidation. In addition these reactive species can damage important molecules such as DNA by oxidizing or damaging DNA proteins.

In normal conditions the human body contains free radicals and oxidants. Even through breathing body acquires oxidants. However, the important point is to provide a balance between oxidants and antioxidants in the body. Through this balance, it is possible to observe oxidative stress, which is caused by uncontrollably increased oxidants (Pérez & Aguilar, 2013).

1.3 Polyphenols

Polyphenols are biological molecules that have a wide range of sources and health benefits. They have a chemical structure that is composed of phenolic

structures. This phenolic structure can be expressed with organic compounds and aromatic rings. Aromatic rings two different groups in terms of chemical structure and they are 6 carbonated molecules. One is hydroxyl group, OH, and the other one is phenyl group, C_6H_5. Polyphenols are mainly plant derived organic molecules and they have many different sources.

In recent years, many epidemiological studies and nutrition research show that the consumption of polyphenol rich foods such as fresh vegetables and fruits affects human health positively. Scientific data about polyphenols, particularly about their effects on the cardiovascular system, has increased significantly. Their antioxidant capacities and properties have been studied widely and the principle of their protective role is the further step of oxidative stress (Scalbert, Johnson, & Saltmarsh, 2005). In nature there are many different polyphenols. The more recent research also shows that there are variety of foods other than fresh foods that are a good sources of polyphenols and cocoa is one of them (Andújar, Recio, Giner, & Rios, 2012).

1.3.1 Source of Polyphenols

The most widespread sources of polyphenols, which are commonly found in vegetal foods and frequently consumed in daily diet, are foods such as pomegranates, grapes, cranberries, tea and cocoa. Research gives important information about the antioxidant content of the different foods. Tables 1.2 and Table 1.3 present the Catechin/Epicatechin concentrations and polyphenol and antioxidant content found in food, respectively.

Table 0-3 Flavanol Concentrations Found in Variety of Food*

Source	Flavanol Content, mg/kg or mg/L
Chocolate	460–610
Beans	350–550
Apricots	100-250
Cherries	50-220
Peaches	50-140
Blackberries	120-140
Apples	20-120
Green tea	100-800
Black tea	60-500
Red wine	80-300
Cider	30-50

*This table is taken from Corti, Flammer, Hollenberg and Lüscher (2009)

According to Table 1-3, cocoa beans and chocolate are ranked in the first 3 foods in terms of their flavonol content. Green tea, red wine, and apricots also have the high level of antioxidant content. When the portions of these foods are considered, consumption of 10 g chocolate provides almost the same flavonol content as 200 ml of green tea. Cocoa and thus chocolate consumption covers a high amount of daily flavonol requirement because it is common food stuff available everywhere and at any time.

It is important to have high flavonol content for food sources because it will already be reduced during several processes that will be applied during the production steps of food products such as heating, drying, etc. The study below

gives information about total phenolic and total flavonoid contents of cocoa, black tea, green tea and red wine (Table 1-4).

Table 0-4 TPC and TFC content of cocoa, black & green tea, and red wine*

	Total Phenol (mg of GAE/ mg extract)	Total Flavonoid (mg of ECE/ mg extract)
Cocoa	611	564
Black Tea	124	34
Green Tea	165	47
Red Wine	340	163

*This table is taken from Lee et al. (2003)

Lee, et el. indicate that total flavonoid ECE amount is the highest in cocoa. Table 1-3 postulates that cocoa has a much higher level of antioxidants than other well-known antioxidant sources such as red wine and teas. This difference comes from the EC and C content of cocoa. Although teas and wine contain many phenolics, the flavonoid content in cocoa exceeds their average because of its high EC content. (Lee et al., 2003)

1.3.2 Types of Polyphenols

There are many different antioxidants found in nature and there are also some additional synthetic types. According to nutritional facts, the daily intake of polyphenols as an antioxidant source for the body is higher than the daily vitamin intake. When the amount for polyphenols is 1g per day, this amount decreases to 100 mg per day for vitamins (Polyphenols, 2014).

Polyphenols have different types. More than 8000 phenolic structures are currently known, and among them over 4000 flavonoids have been identified. Polyphenols can be classified according to their chemical structure, origin and function. These subgroups are explained in the Figure 1-4 (Liu, 2004).

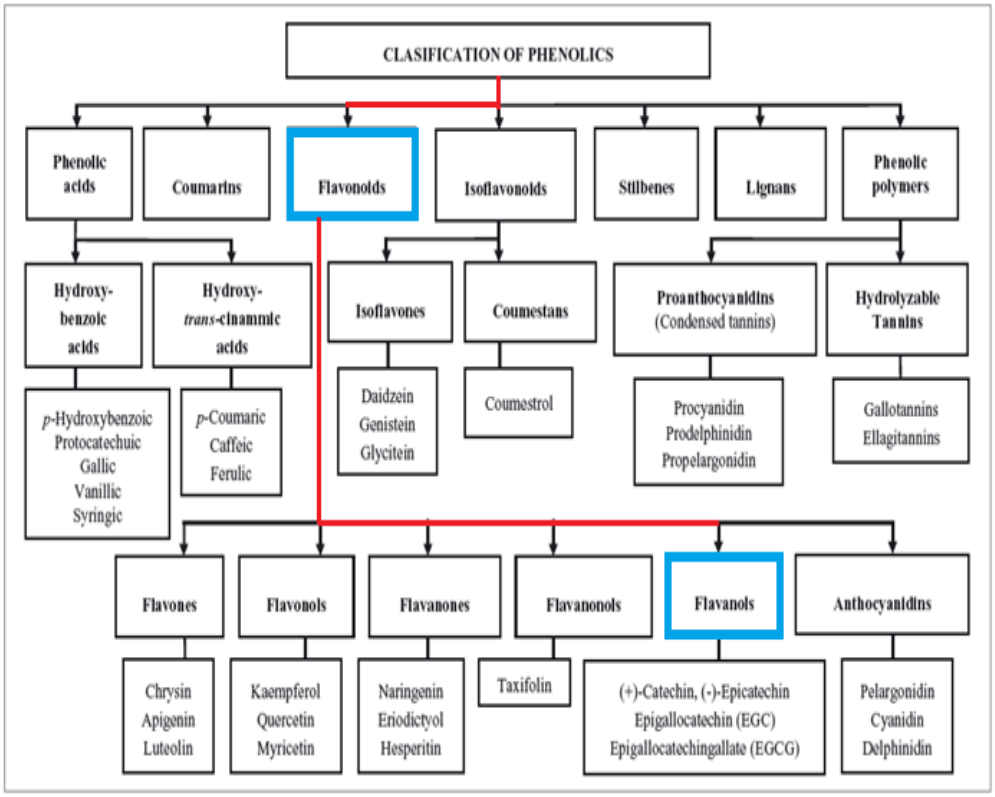


Figure 0-4 Classification of phenolics adapted from Liu (2004)

Flavonoids and its subgroup, flavanols, are the polyphenols that are found in cocoa. They have specific examples: (+)-catechin and (-)-epicatechin. The other common subgroup is phenolic acids. Gallic acid is an example of phenolic acids which is commonly used as a standard phenolic acid in several assays.

According to Andújar et al. (2012), there are 380 different chemical structures known in cocoa. The amount of polyphenols in cocoa is very high and this causes a very bitter taste in the cocoa beans. This intense bitter taste is one of the reasons why cocoa beans are not able to be consumed directly. In order to

make it more edible and give it a delicious taste, cocoa beans are exposed to different processes. At the end of these processes, they lose 90% of their polyphenol content. However, even after this loss, cocoa powder still has remarkable phenolic compounds in it.

Cocoa has different phenolic compounds; catechins, anthocyanidins, and proanthocyanidins (Andújar et al., 2012), The most known and highest bioavailable type is catechins. They are (-)-epicatechin, (+)-catechin, (+)-gallocatechin, and (-)-epigallocatechin. Among these, (-)-epicatechin has the highest level; on the other hand, others occur in lower levels. In addition to this, bioavailability results show that (-)-epicatechin presents at its highest level in plasma 2 hours after consumption and that 20% is excreted from the body by urine.

Cocoa polyphenols show differences in amount dependent on processing. In addition, the cocoa polyphenols presence is also related to (Stahl et al., 2009) cocoa bean type, harvesting conditions, fermentation conditions, drying and roasting. Among these treatments, fermentation and roasting have a decreasing effect on cocoa's phenolic content. In addition, applying alkalization treatment during process also reduces the phenolic content and damages the (-)-epicatechin content, which is the most important and highly unstable phenolic compound. However, this treatment gives better organoleptic properties to cocoa powder, such as darker color, indicating a higher amount of cocoa in cocoa products, and lower bitterness. Because of these reasons, producers prefer to use alkalized cocoa powder in their factories. This research will examine both natural and alkalized cocoa and their products, which will give information about this processing effect on cocoa polyphenols.

1.4 Flavonoids

Polyphenols can be mainly divided into two parts, Phenolic acids and Flavonoids. Flavonoids have the C6–C3–C6 general structural backbone in which the two C6 units (Ring A and Ring B) are of phenolic nature, as shown below. Flavanols or flavan-3-ols are often commonly called as catechins. Catechin is the isomer with *trans* configuration and epicatechin is the one with *cis* configuration. Each of these two configurations has two steroisomers, i.e., (+)-catechin, (−)-catechin, (+)-epicatechin and (−)-epicatechin. (+)-catechin and (−)-epicatechin are the two isomers mainly found in food plants. Figure 1-5 represents the basic chemical structure of the flavonoids (Bravo, 1998).

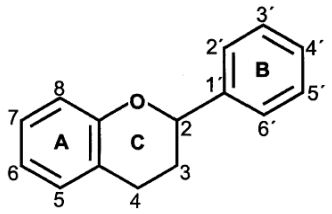


Figure 0-5 Basic chemical structure of the flavonoids

Flavanols are found in many fruits, particularly in the skins of grapes, apples and blueberries. Monomeric flavanols catechin and epicatechin and their derivatives such as gallocatechins are the major flavonoids in tea leaves and the cacao beans, regardingly also in chocolate.

Cocoa is a fruit which contains a high amount of flavanols, especially (−)-epicatechin & (+)-catechin. The chemical structures of major cocoa phenolics are shown in Figure 1-6 (Andújar et al., 2012).

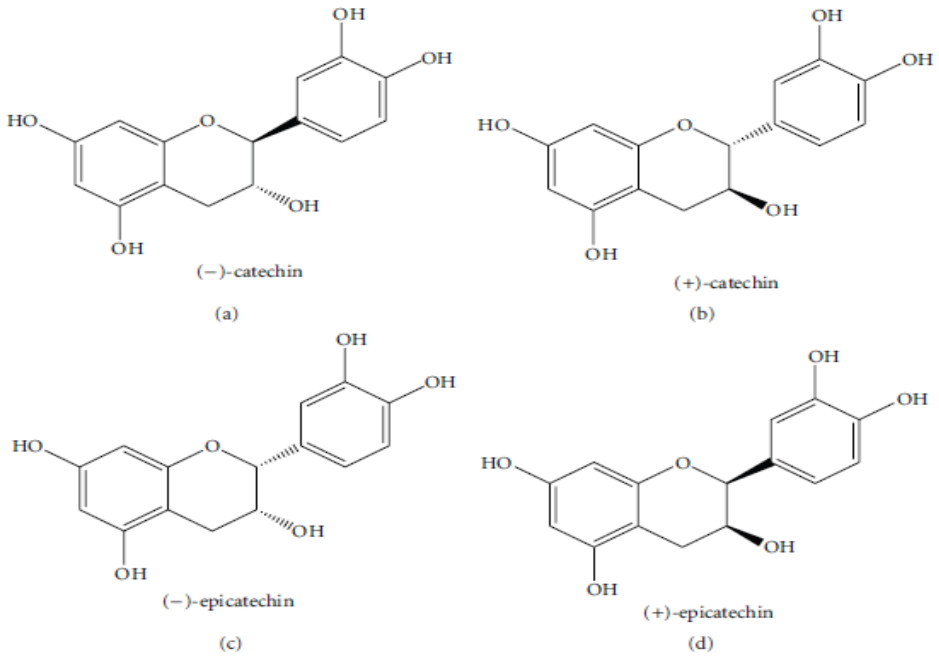


Figure 0-6 Chemical structure of major cocoa polyphenols

1.5 Cocoa and Health

For centuries, cocoa has been known not only for its good taste but also for its proposed health effects. However, the recent discovery of biologically active phenolic compounds in cocoa has changed this perception and stimulated research on its effects on anti-aging, blood pressure regulation, and atherosclerosis. Epidemiological data demonstrate that the regular dietary intake of plant-derived foods and beverages reduces the risk of coronary heart disease and stroke and is inversely associated with the risk of cardiovascular disease. The first evidence of a similar effect of cocoa was obtained in Kuna Indians, a native population living on islands off the coast of Panama.

Kuna Indians were noted to have a low risk of hypertension. In addition, aging people in this population, contrary to the general knowledge, were not

observed to have problems with high blood pressure. When they were migrating through different areas during the years, it was observed that these health properties decreased. This discrepancy indicated that being especially healthy was not a property related to their genetic specialties, but rather it was related to environmental conditions. When their daily habits were analyzed, it was observed that they had different eating habits when compared with other people living in the same area. Kuna Indians were eating 10 times more cocoa than the other urban population living in the same geological area. Research indicated that regular daily cocoa consumption could be the main reason this healthy population had lower risk of hypertension and high blood pressure. The most important beneficial health compound is (-)-epicatechin in cocoa. The health effects are shown in Figure 1-7.

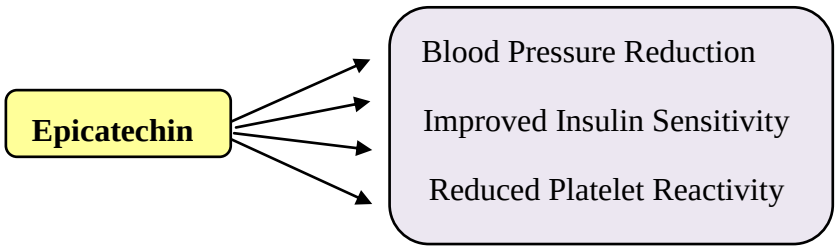


Figure 0-7 Health effects of (-)-epicatechin in cocoa

1.5.1 Cocoa and Blood Pressure reduction & Cardiovascular Health

Many research studies revealed that there is a relation between cocoa consumption and blood pressure balance in the body. One of them indicates that consuming 6 g of chocolate, which contains 30 mg of cocoa flavanols, each day regularly for 4-5 months, provides significant health benefits. The main contribution is related to lowering the blood pressure, by 2-3mmHg (Davison et al., 2010). Zomer et al. (2012) indicate that if chocolate is regularly and daily consumed it will help prevent cardiovascular diseases (Zomer et al., 2012).

It has been stated by invivo test that there is positive effect between cocoa and blood pressure. These effects compared on people regularly consuming chocolate and non-consuming control people. The analyses have been applied over a five year period over 15 years. The consumer group had lower blood pressure than the control group at the end of the study, and the difference between the two groups was significant (Zomer et al., 2012).

1.5.2 Cocoa and Cancer

Cocoa helps to reduce the oxidative stress in the body by providing free radical scavenging activity. Cocoa polyphenols have a protective effect against active oxygen radicals, which have a damaging effect on cells. The cocoa flavonoids prevent DNA damage caused by oxidation in the cells. This effect also protects from the cancer, which occurs by cell damage. According to Latif (2013), *in vitro* studies that show that cocoa has an inhibitory effect on cancerous cells. The mechanism behind these suggestions is related to the anti-genotoxic effects of catechin, epicatechin, epigallocatechin, and gallocatechin. Polyphenols have protective effects on nitrosamine, aflatoxin, and tobacco smoke and prevent DNA damage shown with *in vitro* and *in vivo* studies. In addition many studies

show that before tumor inducement treatments results are better when compared with treatments have been applied after tumor occurrence was completed. The studies show that the molecular mechanisms behind the these significant cancer preventive and antiproliferative effects of cocoa and cocoa products are usually related with their polyphenol content.

1.5.3 Objectives of the study

Antioxidants have a significant impact on human health. In nature, there are different sources of antioxidants and also there are different antioxidant subgroups, such as vitamin C & E, some enzymes such as SOD and catalase, and phenolic sompounds such as flavonoids and phenolic acids. The bioavailability of these antioxidants differs and some polyphenols have significant advantages in terms of bioavailability. Epicatechin, has the highest bioavailability capacity among the catechin types of polyphenols. In addition to this, it is also known that epicatechin has various health benefits on human.

Cocoa is one of the best sources of antioxidants and it is known to have a high epicatechin content. However, there are researches showing that the epicatechin in cocoa is not a stable compound and it is easily decomposed and converted to another phenolic structure upon processes (Corti et al., 2009). In addition to fermentation, other processes such as roasting and alkalization during cocoa powder production has an adverse effect on polyphenols in cocoa. This may cause a reduction in the quantity of polyphenols or a transformation of some phenolic molecules into other monomers.

In this study, cocoa is investigated from this perspectives because of its important antioxidant content. Cocoa and its polyphenols are studied in this project in order to provide better understanding about whether natural and alkalized cocoa and their cakes differ in terms of antioxidant capacity and content. In order to obtain more accurate results, four different analytical methods and chromotographic analysis were used in this study.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

For this research Natural and Alkalized cocoa were kindly donated by Altınmarka. All chemicals used for experiments were purchased from Sigma & Merck as presented in Table 2.1. The catechin and epicatechin measurements were done in Reverse Phase HPLC (Shimadzu UFLC- with C18 column)

Table 0-1 Chemicals Used for Experiments

Chemical Materials	Brand	Chemical Materials	Brand
DPPH	Sigma	Acetonitrile	Sigma
Trolox	Sigma	HPLC water	Merck
ABTS	Sigma	Formic Acid	Merck
Trifluoroacetic Acid	Merck	Methanol	Merck
Gallic Acid	Merck	Ethanol	Merck
Catechin	Sigma	Quercetin	Sigma
Tetrahydrofuran	Merck	Acetic Acid	Fluka
Sodium Carbonate	Riedel-de Haen	Folin Ciocalteu's	Merck
Epicatechin	Sigma		

The other materials that were used in the preparation and experimentation of samples were sourced from the laboratory of the Food Engineering Department. The (+)-catechin and (-)-epicatechin contents of samples were analyzed using by HPLC. All chemicals used in experiments were HPLC grade which means suitable for chromatographic analysis.

2.2 Methods

2.2.1 Sample Selection

In this study, natural and alkalized cocoa and their cocoa cakes were examined as displayed in Figure 2-1. The samples are named as NC for natural cacao, AC for alkalized cocoa, NCC1 and NCC2 for natural cocoa cakes, ACC1 and ACC2 for alkalized cocoa cakes.

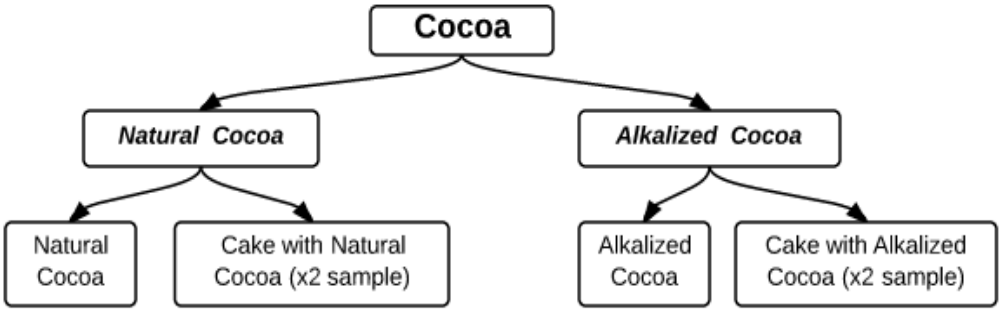


Figure 0-1 The sample selection

Cake is used as product of cocoa samples. The reason behind selecting cake as a cocoa product is that it is a very common product, which is less complicated and easier to produce at home than cocoa cookies, and it is more preferable than the direct cocoa drinks. Nowadays many bakery companies both in Turkey and around the world have cake products. On the other hand, it is a very commonly consumed baked snack that is easy to prepare at home and is

preferred to prepare as a dessert. For all these reasons, cocoa cakes were chosen to be prepared as the cocoa product samples. Cake samples were prepared both from natural and from alkalized cocoa.

2.2.2 Sample Preparation

Cocoa samples were obtained in powdered form and used directly in defatting process. On the other hand, cake samples were prepared and grinded for defatting process. The homemade cake recipe was used for cooking natural and alkali cocoa cake samples. In order to optimize the cocoa percentage in the cakes, the products in the market were investigated for their ingredients and the percentages. Commercially available cocoa cakes contain around 2-3% cocoa. According to some homemade recipes, the average cocoa content of homemade cakes are around 5-7%. Therefore the cocoa content of the recipe was optimized at 6.28%, which is quite close to the homemade recipe. 15 g egg and 42.88 g sugar were mixed as the first step of production and 21 g oil was added and mixed for 30 sec. (see Table 2.2)

Table 0-2 The list of ingredients and their amounts

Ingredients	Amount (g)	Ingredients	Amount (g)
Egg	15.00	Baking Soda	0.75
Vanilla	0.53	Cocoa	10.50
Salt	0.29	Flour	38.75
Sunflower oil	21.00	Water	37.50
Sugar	42.88		

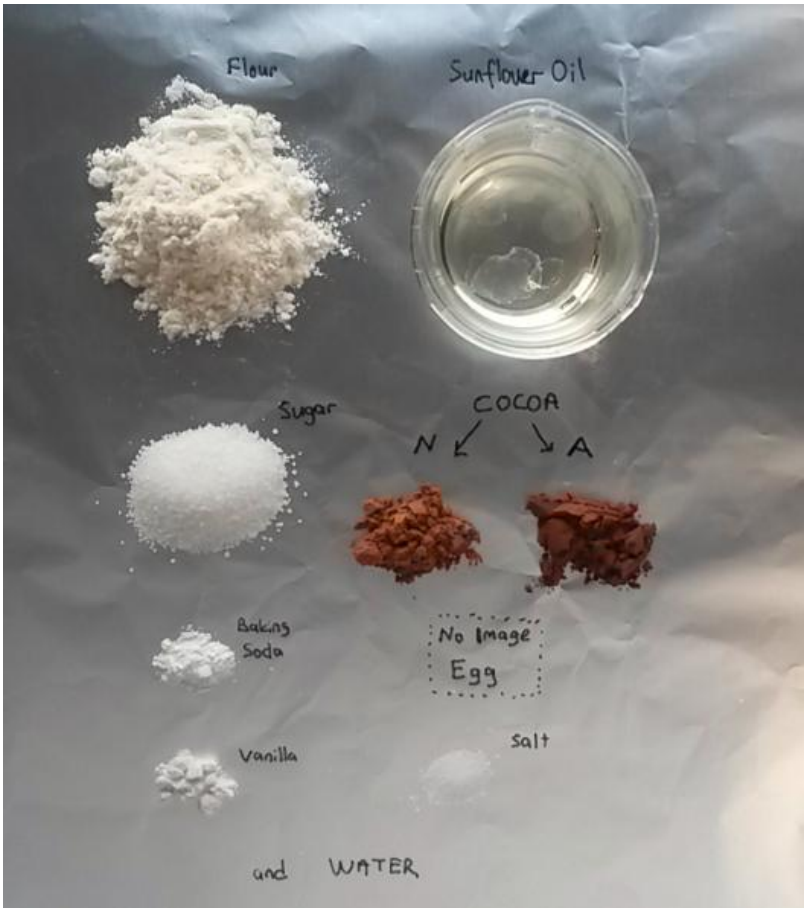


Figure 0-2 Cocoa cakes ingredients

Flour and water were gradually added to the egg and oil mixture. Finally 10.5 g natural cocoa, baking soda and salt were added and mixed until a homogeneous mix was obtained. The cake samples were cooked for 23 minutes at 175 ℃ in a pre-heated conventional oven.

Figure 0-3 Natural Cocoa cakes samples after cooking

The cake samples are shown in Figure 2-3 were weighed before and after cooking; and by this method water loss during the cooking period is calculated. Then samples were ground until a powder was obtained. The aim of grinding is to increase the surface area and consequently increase extraction efficiency.

2.2.3 Defatting of Samples

In this process all the natural, alkalized cocoa and cake samples were defatted before extraction. In literature, cocoa normally has 10-12% fat content and this fat contains almost no polyphenols. In order to obtain more accurate results this fat should be removed and hexane was used as extraction solvent.

5 g of each sample was mixed with 50 ml hexane and mixed well for 30 seconds. Then the slurry mixture was placed in the shaker set to 110 rpm 25 ^{0}C and shaken for 60 minutes. After a while, 2 separate layers form and the solid part is precipitated. Thus the clear hexane and oil solution is easily pipetted out. It was filtered with filter paper and the same process was duplicated respectively for each sample: adding hexane, vortex for 30 sec., shaking, piping out, filtering and drying. The hexane is very volatile and the precipitated part was dried at room temperature. At the end of this process, 85.57 g natural and 76.40 g alkalized non-fat cocoa were obtained from 100 g cocoa and 66 g natural and 65 g alkalized non-fat cocoa were obtained from 100 g cocoa cake.

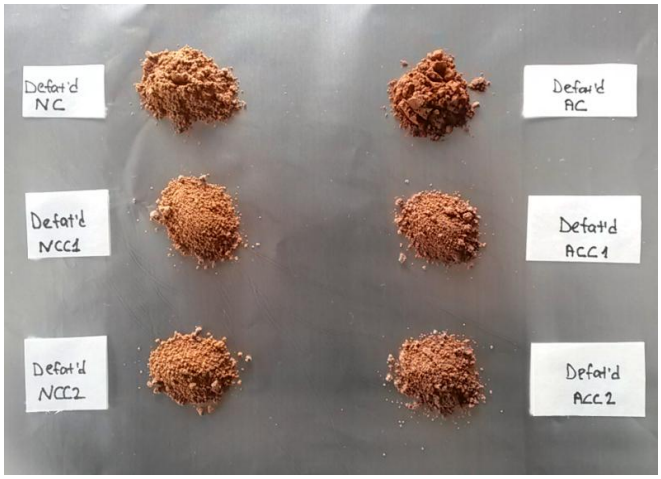


Figure 0-4 Defatted Natural & Alkalized Cocoa and Cakes

2.2.4 Extraction of soluble phenolic compounds

Similar extraction processes in the literature were examined in order to determine the solvents appropriate for the extraction process of cocoa samples. Adamson used a mixed solvent solution of Acetone/Water/Acetic Acid (70:28:2) in order to extract polyphenol (Adamson et al.,1999). On the other hand, some studies only used acetone or an acetone-water mix as an extraction solvent. Therefore Acetone/Water/Acetic Acid (70:28:2). Acetone/Water (70:30) and Distilled Water (100) solvents were compared with each other in terms of their antioxidant capacities and the experiment results showed that the Acetone/Water/Acetic Acid (70:28:2) was the most suitable solvent, in parallel with the results of Adamson. (Adamson et al.,1999)

The polyphenol extraction steps are:

- The 4 g defatted samples were weighed.
- 20 ml solvent was mixed with defatted sample within a plastic test tube (Ratio 1:5)

- Test tube was put to vortex device for 30 sec.
- Well mixed solution was placed into the Shaker overnight at 110 rpm 30 ℃
- After the shaking process, it was filtered by very fine filter paper.
- The same steps were repeated.

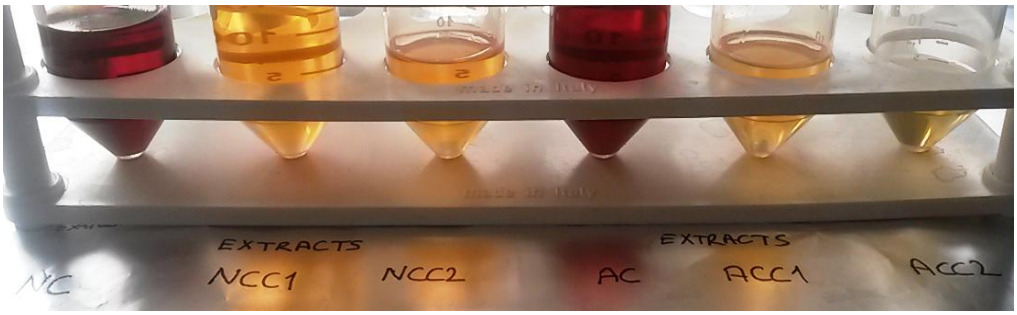
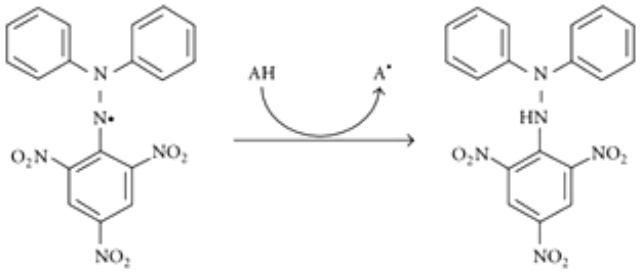


Figure 0-5 Extract of Natural and Alkalized Samples

After the solvent was optimized as Acetone/Water/Acetic Acid (70:28:2), all the samples were extracted using the same solvent

2.2.5 Determination of antioxidant capacity DPPH (2.2-diphenyl-1-picryl-hydrazyl) Radical Scavenging Activity

In this experiment, the free radicals were reduced by DPPH solution.In this study the Blois (1958) and Brand-Williams, Cuvelier and Berset's (1994) methods were modified and used.



(Purple) (Yellow)

Figure 0-6 Mechanisim of DPPH method

The DPPH stock solution was prepared by adding ethanol until the absorbance became 1.4 with 517nm wavelength. After preparing the stock solution, it needs to be kept in the dark since it can be easily affected by the light. The extract samples were diluted as 1/2, 1/3, 1/4 1/5 1/6 and 1/8 and 0.1ml of extract was added to the test tube. Then 1.4 ml of DPPH solution was added to the top of the test tube and mixed. The well mixed test tube was kept for 20 min in the dark. Finally absorbance was measured at 517 wavelength and data were recorded.

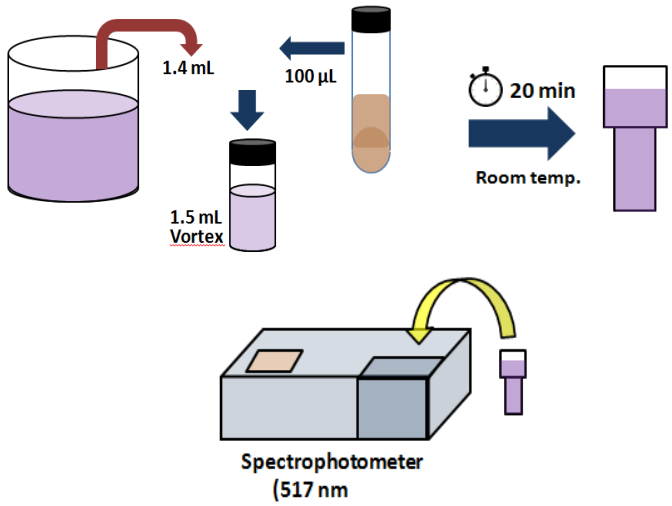


Figure 0-7 Flow Chart of DPPH

All the same procedures were applied for all extract samples with different dilution ratios. After the experiment %RSA and IC 50 value were calculated.

Radical Scavenging Activity (%) = {[OD Control-OD sample]/OD Control} x100 = [(Ao-A1)/Ao] x100

Ao : Abs of DPPH with ethanol

A_1 : Abs of DPPH with extract conc.

2.2.6 Determination of the Total Phenolic Content

The total phenolic content of samples was measured by the Folin–Ciocalteu procedures according to the method of Singleton and Rossi's (1965) Method. Gallic acid is used in order to obtain standard curve. Similar to DPPH method, all the samples and Gallic acid standard were diluted. At the beginning 0.15 ml Standard /or Extract solution and 3 ml %2 Na_2CO_3 were put to test tube mixed vigorously. After 3 min 0.15 mL of 50% Folin- Ciocalteu phenol reagent was added to the mixture and the tubes were subjected to vortex. The mixture was allowed to stand in the dark at room temperature for 30 minutes. After the incubation at room temperature, the absorbance was measured at 750 nm using a spectrophotometer. Results were expressed as milligram per gram of Gallic acid equivalents (GAE). All the steps were applied 4 times for each sample.

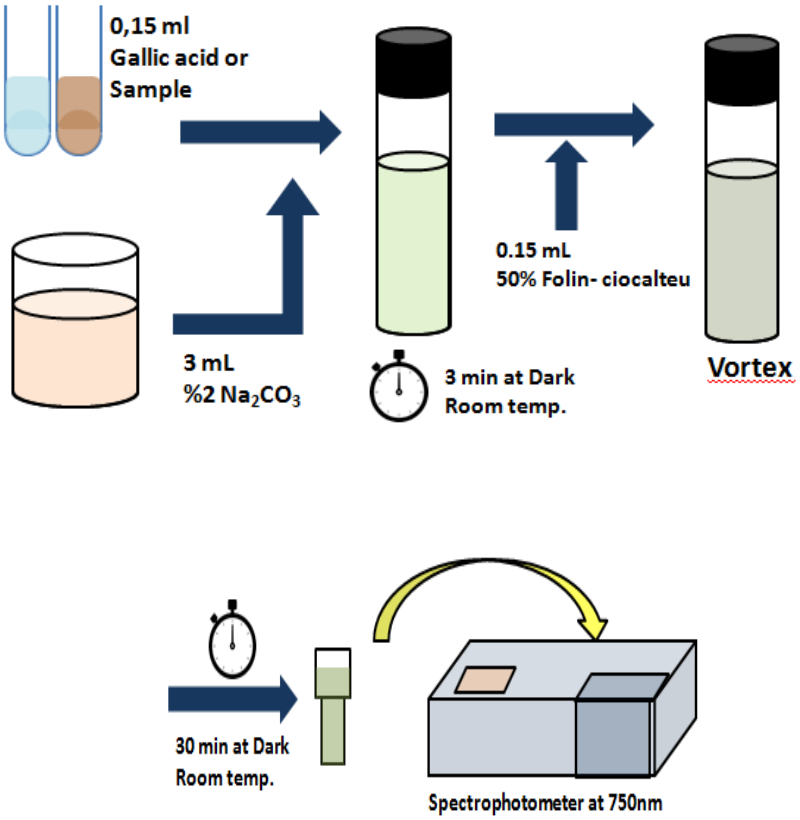


Figure 0-8 Flow Chart of Total Phenolic Content Assay

2.2.7 Determination of the Total Flavonoid Content

The total flavonoid content was measured by the aluminum chloride colorimetric assay (Zhishen, Mengcheng, & Jianming, 1999). 1 M NaOH. 5% $NaNO_2$ and 10%$AlCl_3$ solutions were prepared. All the extract samples and quercetin standard were diluted. 0.5 ml of diluted sample or standard solutions of quercetin were added to the test tube that contains 2 ml of distillated water . After 5 min rest in the dark at room temperature 0.15 ml 10%$AlCl_3$ solution was added and kept in dark for 1 minute. At the 6^{th} min. 1 ml 1M NaOH solution was added and the total volume was made up to 5 ml by adding 1.2 ml distillated water. The solution was mixed well and the absorbance was

measured against prepared reagent blank at 510 nm. The total flavonoid content was expressed as mg quercetin equivalents (QE) / 1 g dry plant material. Samples were analyzed for 4 times.

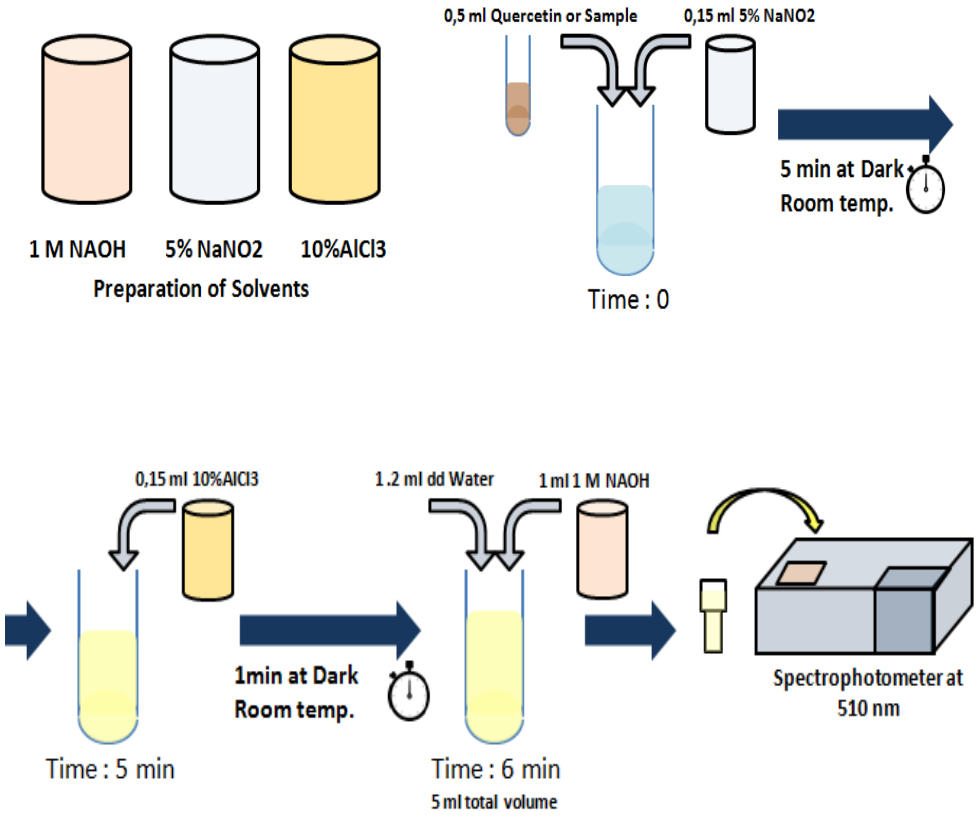


Figure 0-9 Flow Chart of Total Flavonoid Content Assay

2.2.8 Determination of Trolox Equivalent Antioxidant Activity

For ABTS assay, the procedure followed the method by Arnao, Cano and Acosta (2001) with some modifications. The stock solutions included 7 mM $ABTS^{\cdot+}$ solution and 2.45 mM $K_2S_2O_8$ (Potassium persulfate) solution were prepared and kept for 16 hours in the dark at room temperature. Then, the ABTS solution is diluted by adding 60 ml methanol to 1 ml $ABTS^{+}$ solution until obtaining 1.1±0.02 absorbance at 734 nm. At the last step of the method 0.1 ml of cocoa extract was mixed with 1ml of the $ABTS^{+}$ solution. The mixture was left for 6 min in a dark place and then the absorbance was measured at 734 nm. (Martínez, Torres, Meneses, Figueroa, Pérez-Álvarez, & Viuda-Martos, 2012). Results are expressed in μM Trolox equivalents (TE)/g drymass, as mean of four replicates.

The percent inhibition was calculated as: Inhibition % = [(A0-A1)/A0] x 100 where A_0 is the absorbance of the ABTS solution and methanol. A_1 is the absorbance of the ABTS with the extract concentrations.

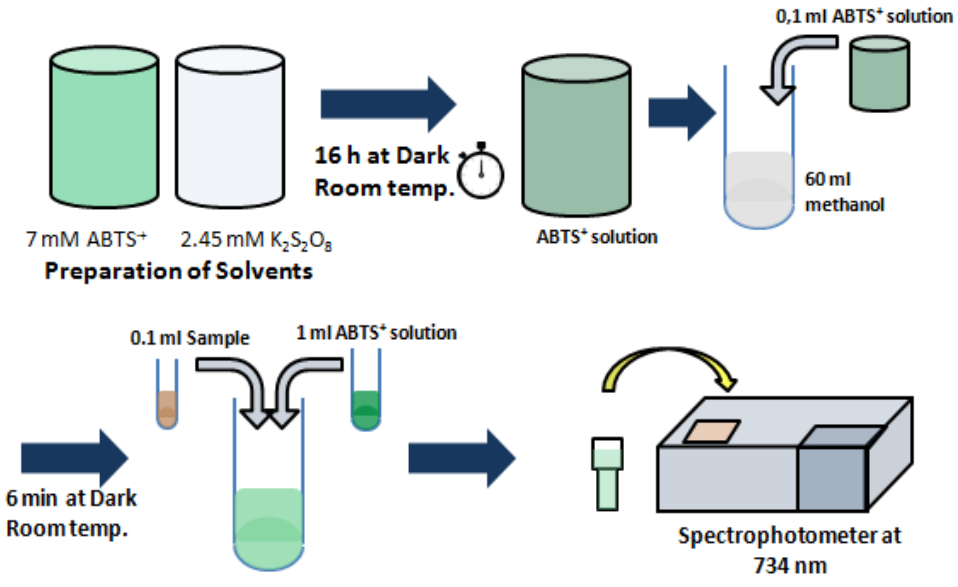


Figure 0-10 Flow Chart of ABTS Assay

2.2.9 Analysis of Phenolic compounds by high-performance liquid chromatography (HPLC)

Catechin and Epicatechin Analyses were done using reversed phase HPLC with C-18 column. In addition for the chromatographic analysis HPLC, rapid reverse phase, is used. The HPLC equipment consisted of a Shimadzu LC-20AD system including DGU-20A5 prominence degasser, SIL-20AHT prominence autosampler, SPD-M20A prominence UV-Vis photodiode array detector, CTO-20A prominence column oven. The column was an Agilent Zorbax SB-C18 (250 mmx 4.6 mm, 5 μm) is used for chromatographic separation. Diode Array Detector (280nm) was used. Separations were performed by a series of linear gradients of B into A at a flow rate of 1 mL/min and of 0.5 mL/min. For the solvents binary mobile phase is used and it was consisted of two solvent solutions A and B. A is water: tetrahydrofuran: trifluoroacetic acid (98:2:0.1, v/v/v) and this is polar solvent, B is acetonitrile**:** trifluoroacetic acid (99.9: 0.10, v/v). Data was processed on an Intel pentium IV PC computer by using LC Solution Programme.

After the sample preparation, the HPLC device was set according to the conditions which are available in Appendix. The binary system phases were (A) water/ THF/TFA (98:2:0.1 v/v/v) and (B) acetonitrile with 0.1% TFA.

Figure 0-11 HPLC device used for analysis

The HPLC system was equipped with a binary gradient pump a sample injector. A column oven, a photodiode array detector and a degassing system and driven by software. Five microliters of the diluted extract was injected into the UPLC system and separated by an Acquity HPLC C18 column with the help of solvents. The flow rate of solvents is important in order to provide polar and non-polar molecule selection. The standards are (-)-epicatechin and (+)-catechin and they are analyzed with serially diluted extracts. The different concentrations of the standards give different area under the curves. According to this information, standard curves of EC and C have been drawn and the equations were obtained.

The measurements of the samples were conducted with different concentrations and then the proper concentration was decided in the standard curve range. According to the area under the peak that is obtained at the same time with the standard is used in the standard curve equation and the result gives the equivalent value for EC and C. The flow rate of the solvent, the oven temperature and the pump should be arranged and controlled during the measurements.

CHAPTER 3

RESULTS AND DISCUSSION

In this study, the unprocessed natural cocoa processed (alkalized) cocoa and cocoa containing cake samples were analyzed for their antioxidant activities, total phenol and flavonoid contents. Antioxidant activities of the samples were analyzed by using DPPH and ABTS radicals scavenging. The total phenolic compound content and the total flavonoid content of the samples were also determined in terms of gallic acid and quercetin standard equivalence, respectively. In addition, HPLC analyses were applied in order to specify the (-)-epicatechin and (+)-catechin content of the samples. Alkaline cocoa samples are denoted as AC and Natural cocoa samples as NC. Cakes produced from alkaline cocoa samples are denoted as ACC and cakes produced from natural cocoa samples are denoted as NCC.

3.1 Defatting Result

The cocoa and cocoa cake samples were defatted using hexane and the results are given in Table 3. In order to calculate the total amount of removed fat, each sample was weighed before and after the defatting process; the percentage of defatted sample per gram sample was calculated and demonstrated in Table3-1.

Table 0-1 Defatting Process of Cocoa and Cocoa Cake Samples

Measurements	AC	ACC1	ACC2	NC	NCC1	NCC2
Initial Sample Weight (g)	30	30	30	30	30	30
Final Sample Weight (g)	22.92	18.7	20.4	25.67	20.6	19.5
Removed Fat (g)	7.08	11.3	9.6	4.33	9.4	10.5
Defatted Sample/Sample (%)	76.4	62.33	68.00	85.57	68.67	65.00

As it is shown in Table 3-1, 30 g samples were used for this experiment. The initial fat content of samples was known; therefore the expected and actual amounts could be compared with each other. Normally, natural and alkalized cocoa samples contain around 10- 12 % fat, which means around 26.4 – 27.0 g nonfat cocoa should be obtained from 30 g cocoa. As indicated in Table 3-1, the 25.67 g defatted NC was obtained from 30 g NC. When it was compared with the expected defatted NC amount, 97.2% defatting efficiency was achieved. Regarding the alkalized cocoa, its efficiency was lower than the defatting efficiency of NC. This small gap between efficiencies can be attributed to measuring errors during the experiment. Similarly, the total removed fat amounts are quite close to each other for cocoa cake samples. The average 19.55 g non-fat alkalized and 20.05 g nonfat natural cocoa cake were obtained from 30 g of cake samples.

The theoretical results and the experimental results are quite close to each other with high efficiency ratios. However, the slight difference between them is due to not only the efficiency but also the loss during the experiments. The filter paper used after the hexane application keeps some residue and this causes the losses. In conclusion, the cocoa and cake samples were defatted with high efficiency by using hexane.

3.2 Solvent Selection

Extraction is one of the most important stages of this study. The higher extraction efficiency enables more accurate results from the next steps. In order to decide which solvent is the most suitable for extraction, 3 different methods were applied to a cocoa sample and extracted with three different solvents.

Table 0-2 Cocoa extract amount for three different solvent

Solvent Name	**Solvent 1**	**Solvent 2**	**Solvent 3**
Solvent Composition	Acetone /Water/ Acetic Acid (70:28:2)	Acetone / Water (70:30)	Water (100)
Cocoa Extract (g/100 g cocoa)	7.52	7.38	8.42

According to Table 3-2 the extract amounts obtained from same amount of defatted cocoa differs. Three extracts were obtained from the same amount of defatted sample. However, this difference does not indicate that the higher amount means better extraction. Solvents may also dissolve non-antioxidant compounds; consequently, this may be misguiding and may affect the analyses adversely.

Different methods have been applied on these three different solvents. According to Adamson et al. (1999), solvent with acetic acid results in higher efficiencies.

The DPPH$^{\bullet}$ scavenging activities of the samples were analyzed to evaluate the antioxidant activity of samples. IC_{50} is calculated according to differing percentage of radical scavenging activity of the extracts in different concentrations. IC_{50} data provides the half maximal inhibitory concentration of the samples and the lower IC_{50} determines the higher antioxidant activity. IC_{50} data for DPPH$^{\bullet}$ scavenging activities results for three different solvent are given in Table 3-3.

Table 0-3 DPPH results for three different solvent

Solvent Type	**IC_{50}**
A:W:AA	0.06
A:W	0.09
W	0.44

A: Acetone; W: Water; AA: Acetic acid

The 50% inhibition of DPPH free radical to a stable compound is achieved with the 0.06 mg/ml extract for solvent 1; 0.09 mg/ml with solvent 2; and 0.44 mg/ml with solvent 3. The same sample was used for each measurement and solvent 1 proved to extract more antioxidant compound from the solution.

In addition to antioxidant activity measurement, the total phenolic content determination was applied for 3 different solvents. The Gallic acid standard curve was constructed and GAE (Gallic Acid Equivalent) data of the samples were determined separately.

Table 0-4 Total Phenol Content for three different solvent

Solvent Type	GAE (mg/100 g cocoa sample)
A:W:AA	3320
A:W	3082
W	505

A: Acetone; W: Water; AA: Acetic acid

It is indicated in Table 3-4 that water provided the lowest total phenolics in terms of GAE. According to Kuhlmann et al. (2005), (-)-epicatechin and (+)-catechin are polar molecules and although the water is polar, the TPC results are quite lower than other solvents because water might have dissolved other molecules such as sugars as well (Kuhlmann et al., 2005)

The total flavonoid content determination was finally applied on 3 solvents. Quercetin was used as the standard for this method and the results were indicated as quercetin equivalence. Quercetin standard curve was constructed by measuring the absorbances at 510 nm using different concentrations of quercetin. Parallel to TPC results, the total flavonoid content in terms of QE value was highest in solvent 1.

Table 0-5 Total Flavonoid Content for three different solvents

Solvent Type	QE (mg/100 g cocoa sample)
A:W:AA	2010
A:W	1840
W	304

A: Acetone; W: Water; AA: Acetic acid

Higher DPPH, TPC and TFC results were measured in acetone/water/acetic acid solvent. For this study, the most suitable solvent was used by modifying Adamson et al. (1999) methods to 70:28:2.

3.3 Extraction Process

In the previous section, three different extraction solvents were used for optimization in accordance with the scientific evidence. Acetone/Water/Acetic Acid (70:28:2) was determined to be the most suitable solvent. The extraction process was applied on cocoa and cake samples using this solvent.

Table 0-6 Extraction Results of Cocoa Samples

Extraction	AC	NC
Concentration of extract (mg/ml)	14.4	17.9
mg Extract per 1 g defatted sample	103.9	128.9
mg Extract per100 g sample	7939	11028
g Extract per 100 g sample	7.9	11.0

Table 0-7 Extraction Results of Cocoa Cake Samples

Extraction	ACC1	ACC2	NCC1	NCC2
Concentration of extract (mg/ml)	46.9	48.3	46.1	47.3
mg Extract per 1 g defatted sample	380.0	405.9	387.4	383.3
mg Extract per100 g sample	23688	27598	26600	24912
g Extract per 100 g sample	23.7	27.6	26.6	24.9

The concentrations of samples were measured by drying and g extract per 100 g sample was calculated using the defatting data as well.

The extraction concentration data has critical importance for further calculations. Therefore stock extract concentrations were applied to serial dilutions in order to obtain the proper ranges for further analysis and kept at +4 ℃ until used.

3.4 DPPH Radical Scavenging Activity

The scavenging capacities of the cocoa and cake sample extracts were measured by their ability to scavenge the stable radicals DPPH (Schinella et al., 2010). For all 6 samples, the antioxidant activity was tested spectrophotometrically, measuring the ability of the cocoa extract to scavenge a stable DPPH• free radical (Arnab, Goyal, & Middha, 2010).

Each measurement was performed four times and the average of these four measurements was used in calculations. The formula given below was used for the calculation of % RSA.

DPPH scavenging effect (%) = (A_0 – A_1) / A_0 x 100

A_0 was the absorbance of the control (Blank)

A_1 was the absorbance in the presence of the sample

A_0 does not contain any extract but only contains solvent and A_1 represents the absorbance of the extract at 517nm (Arnab, Goyal, & Middha, 2010). The RSA% result tables of the samples are demonstrated in Appendix in Table A1-A6.

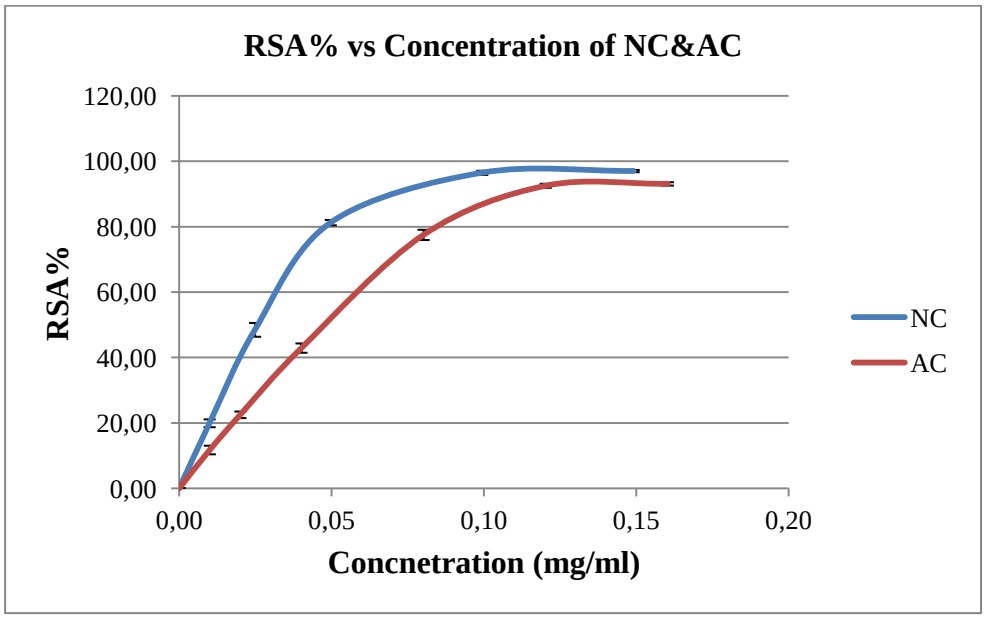


Figure 0-1 %RSA Comparison of Alkalized Cocoa & Natural Cocoa

Figure 3-1 explains %RSA of both types of cocoa in the same graph. The natural cocoa reaches the same % radical scavenging activity faster than alkalized cocoa. The IC_{50} value was calculated in excel as 0.064±0.001 mg/ml for alkalized cocoa and 0.047±0.001 mg/ml for natural cocoa. According to the

literature, there is inverse proportion between IC_{50} value and antioxidant activity. Therefore it can be concluded that the NC has the higher antioxidant activity.

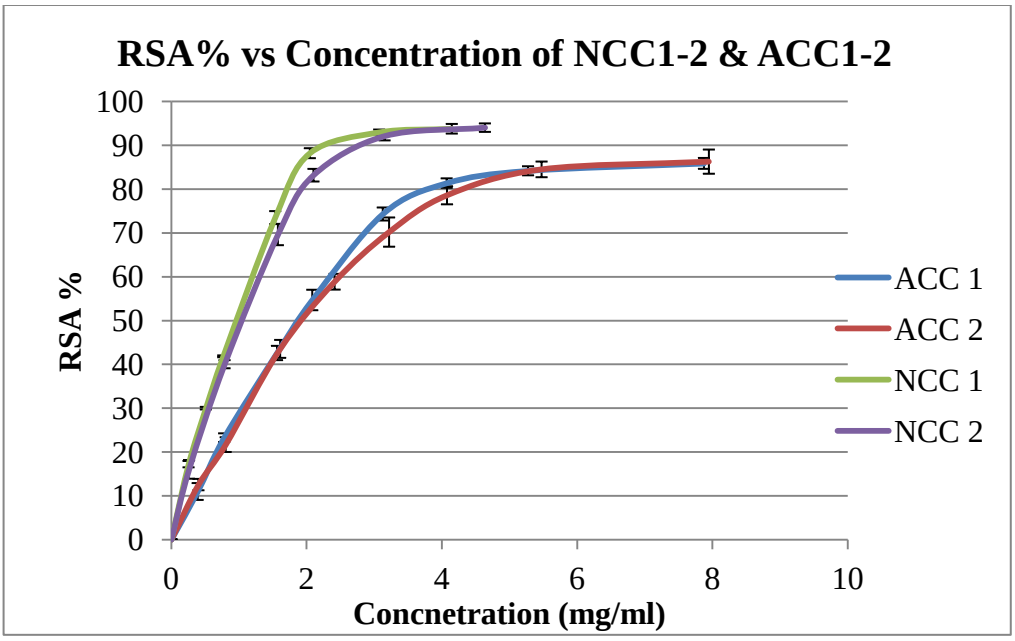


Figure 0-2 %RSA of Natural & Alkalized Cocoa Cake Samples

All the IC_{50} are expressed in Figure 3-3. The unprocessed natural cocoa (NC) has the lowest IC_{50} value with 0.047±0.001 mg/ml. The second lowest IC_{50} value was 0.064±0.001 mg/ml for Alkalized Cocoa (AC). There is an inverse correlation between IC_{50} and antioxidant activity. Lower IC_{50} expresses higher antioxidant activity and regarding to their IC_{50} values, the antioxidant activity of samples can be ordered as following in terms of their DPPH radical scavenging activities.

NC > AC >NCC >ACC

Table 0-8 IC_{50} value of all samples

Sample	IC_{50} (mg/ml)	Sample	IC_{50} (mg/ml)
NC	0.047±0.001	**AC**	0.064±0.001
NCC1	1.376±0.016	**ACC1**	2.742±0.053
NCC2	1.537±0.086	**ACC2**	2.835±0.053

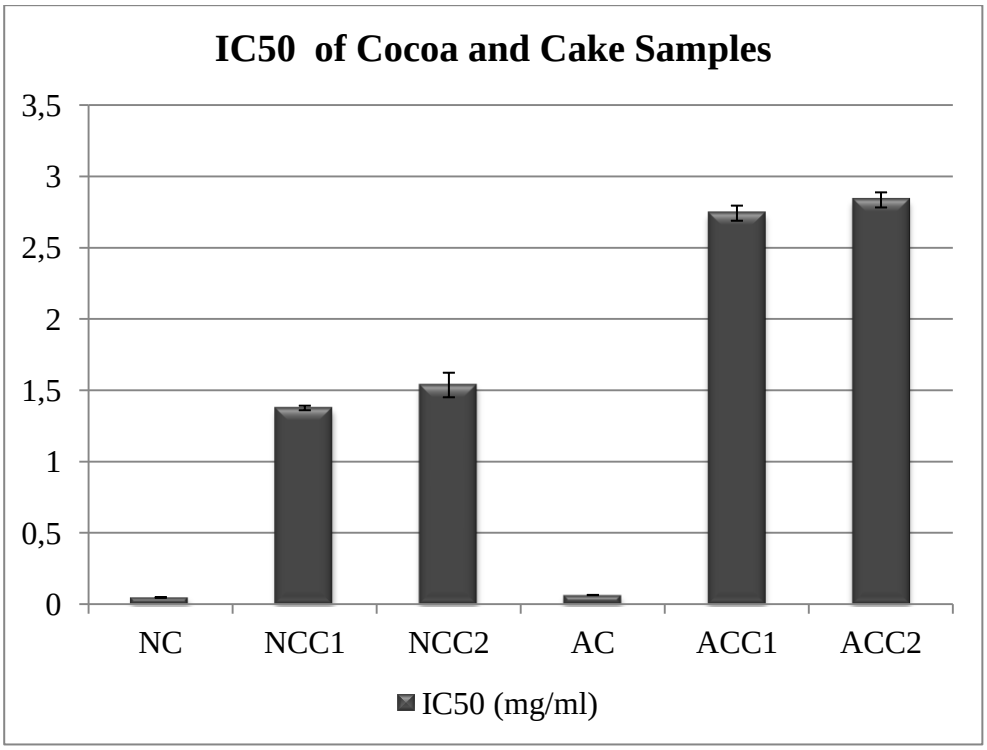


Figure 0-3 The IC_{50} Comparison of Cocoa Samples

In addition, the correlation between samples can be calculated according to IC_{50} value, Table 3-9 indicates that 0.43 mg Natural Cocoa has the same impact as 0.81 mg alkalized cocoa. 5.17mg NCC1 and 10.27g ACC2 have

same effect in terms of antioxidant activity. This shows that two times more alkalized cocoa than the natural cocoa is required in order to obtain the same antioxidant activity.

Table 0-9 Sample required for the same antioxidant activity

Sample	Required amount (mg)	Sample	IC_{50} (mg/ml)
NC	0.43 ± 0.01	**AC**	0.81 ± 0.01
NCC1	5.17 ± 0.06	**ACC1**	11.58 ± 0.22
NCC2	6.17 ± 0.35	**ACC2**	10.27 ± 0.19

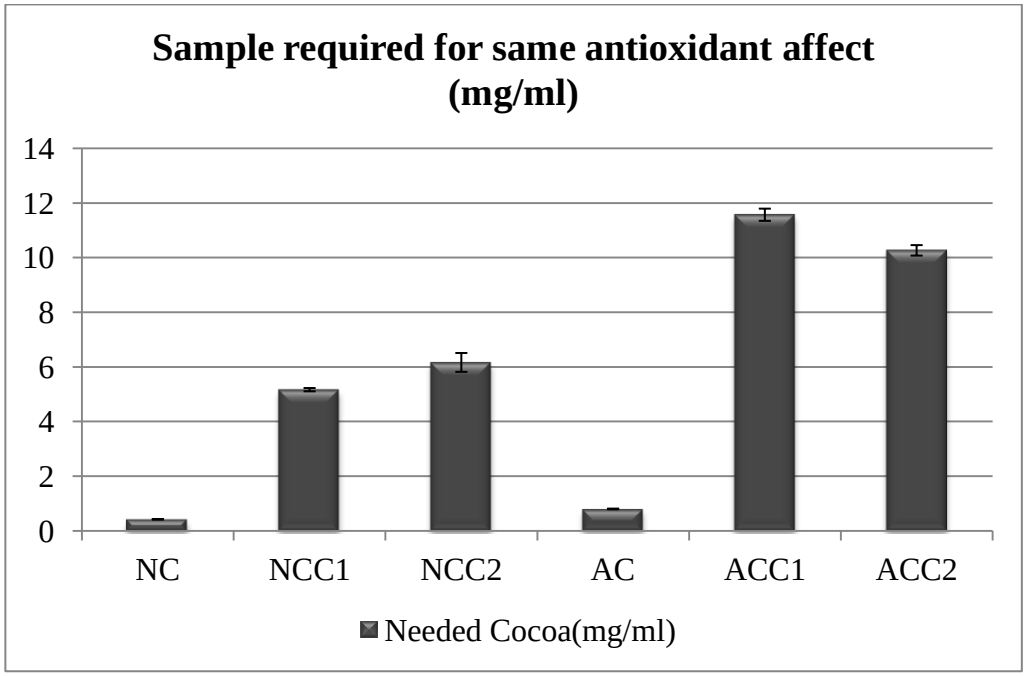


Figure 0-4 Required cocoa or cake amount for the same antioxidant effect

Natural cocoa cakes' effect is two times higher than alkalized cake. Both of these cocoa samples were sourced at exactly the same time from the same supplier and this means the conditions were equal for both. However, the difference ratio between cocoa and their cakes is the same. Even though the cake samples were exposed to the same heat treatment during the baking stage, the antioxidant activity ratio did not change. This may prove that the process after cocoa production did not significantly change or worsen the antioxidant capacity but that the process during cocoa powder production causes significant differences.

3.5 Determination of Total Phenol Content

Folin-Ciocalteu Method was used for the investigation of total phenol content of samples. TPC assay was performed four times for each sample and standard in order to obtain more accurate results with standard deviations. Gallic Acid was used as the standard for obtaining the standard curve. At the end of all the spectrophotometric measurements the equation of standard curve y=57.876x was obtained. TPC assay procedure was applied to all the samples and Gallic Acid Equivalence Phenolic amounts of the extracts were calculated.

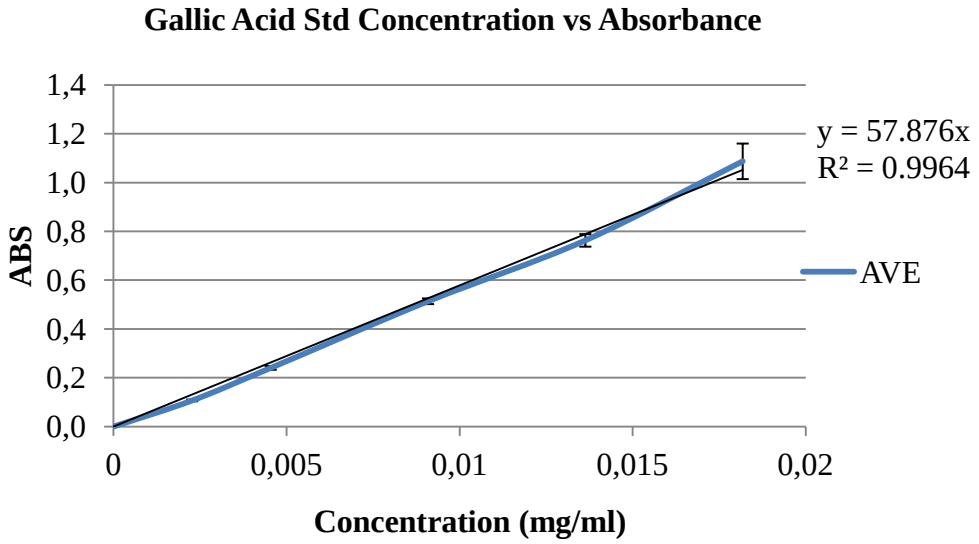


Figure 0-5 Gallic Acid Standard Curve for TPC

Results are expressed as mg of Gallic acid equivalents per 100 g samples. From Table 3-4, it can be concluded that NC contains almost 3 times more phenolic compounds than AC. Similarly. NCC samples contain 2 times more phenolic compounds than ACC samples.

Natural Cocoa has the highest TPC with 4883.84 mg GAE/100g sample. and alkalized cocoa has the second highest TPC with 1708.12 mg GAE/100g sample.

Table 0-10 Results of TPC in terms of Gallic Acid Equivalent

Sample	Extract mg/100 g Sample	GAE mg/100 g Sample	Sample	Extract mg/100 g Sample	GAE mg/100 g Sample
NC	0.4428±0.0347	4883.84	**AC**	0.2151±0.0152	1708.12
NCC1	0.0127±0.0008	339.60	**ACC1**	0.0066±0.0005	158.54
NCC2	0.0131±0.0007	326.97	**ACC2**	0.0065±0.0003	181.68

These results indicate that the total Phenolic content of cocoa significantly decreases during the alkalizing (dutching) process. This decrement is considerably high. The amount of difference can be seen in Figure 3-6.

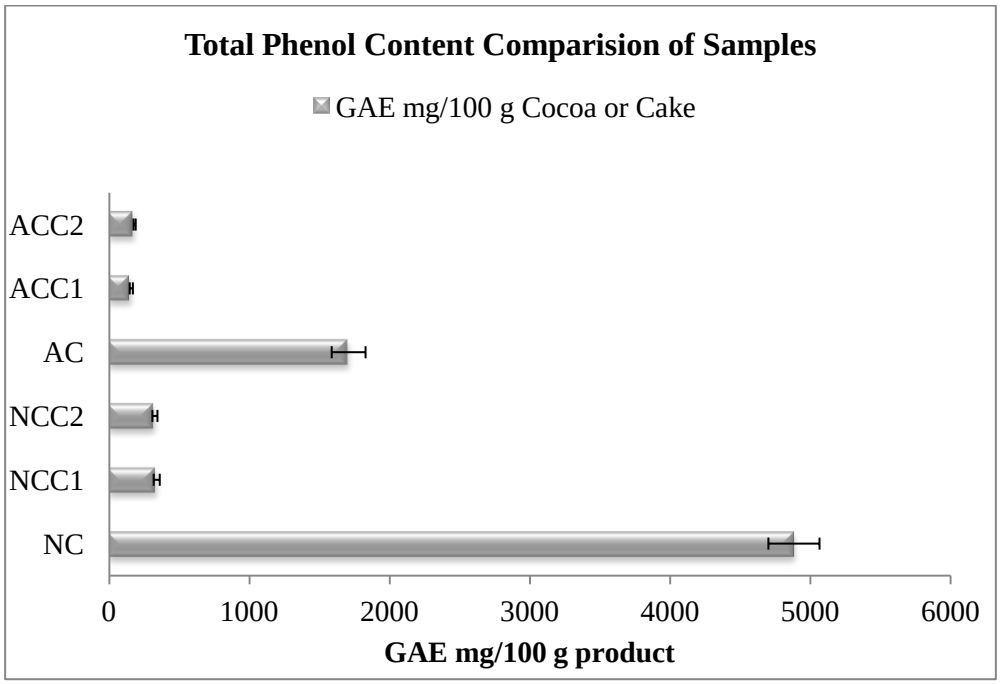


Figure 0-6 Total Phenol Content Comparison of Samples

When these contents are compared with other researches, they are reasonable according to literature. Miller et al. (2006) investigated 19 different cocoa containing products and 3 of them were natural cocoa powders. The total polyphenol content in terms of GAE was found to be between 4500-6000 mg GAE/100 g cocoa. When compared the result in this study is 4883 mg GAE/100 g natural cocoa and this is parallel to Miller's (2006) study.

According to Stahl et al. (2009) homemade chocolate cakes made with processed cocoa contain 1.40-1.50 mg/g sample total polyphenols. In this study for ACC samples GAE mg/ g cake is around 1.50 mg/g (Stahl et al., 2009). This result is the same as the reference study. It is indicated that if natural cocoa is used for cocoa products, the antioxidant content of the product increases.

3.6 Determination of Total Flavonoid Content

Similarly to the TPC procedure Total Flavonoid Content assay (TFC) was applied to all samples. In this experiment Quercetin was used as standard. The standard curve was obtained by measuring each sample four times. At the end of the measurements, the equation of standard curve y=16.818x was calculated and used to express total flavonoid content in terms of Quercetin Equivalence.

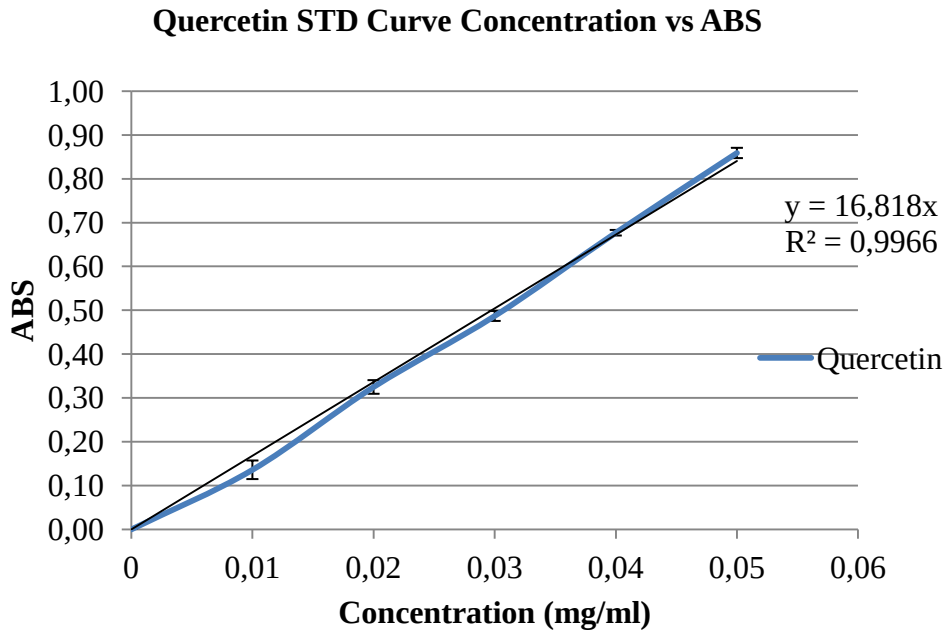


Figure 0-7 Quercetin Standard Curve for TFC

The results are expressed as mg of quercetin equivalents per 100 g sample. Similarly to TPC results Natural Cocoa has the highest TFC with 3155.30 mg QE/100g sample and alkalized cocoa follows with 938.38 mg QE/100g sample.

Table 0-11 Results of TFC in terms of Quercetin Equivalent

Sample	Extract mg/100 g	QE mg/100 g	Sample	Extract mg/100 g	QE mg/100 g
NC	0.286±0.0510	3155.30	**AC**	0.118±0.0230	938.38
NCC1	0.008±0.0009	223.43	**ACC1**	0.004±0.0004	118.26
NCC2	0.008±0.0007	201.30	**ACC2**	0.004±0.0004	133.79

Total cocoa flavonoid content should be less than the total polyphenols content because flavonoids are a subgroup of polyphenols. In addition there should be a similar ratio within the samples in terms of TPC content and TFC content.

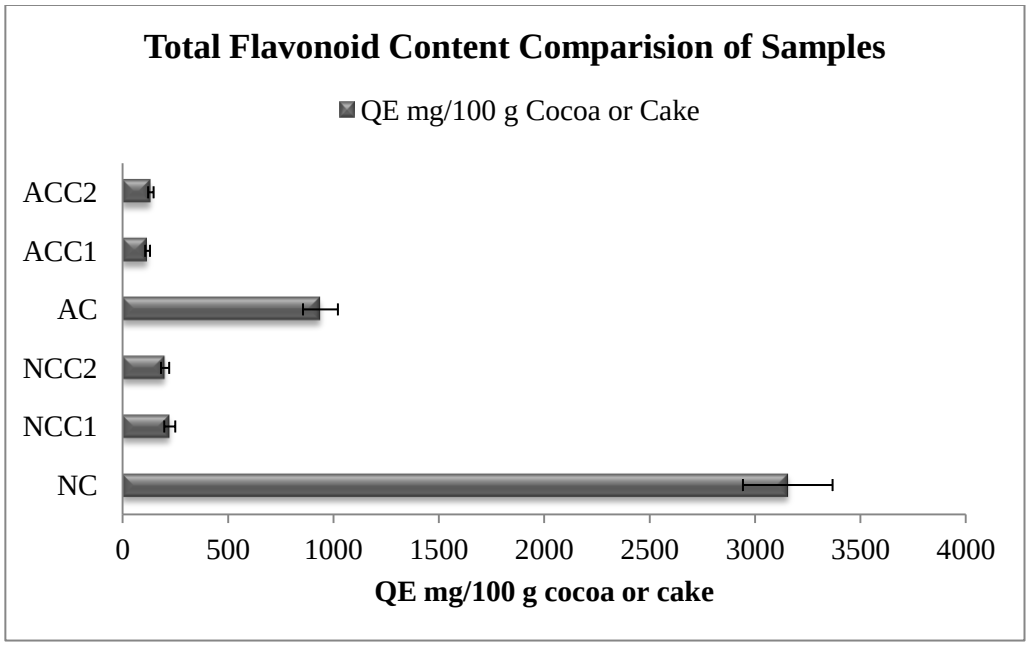


Figure 0-8 Total Flavonoid Content Comparison of Samples

Total flavonoid content of the 6 samples is compared in Figure 3-8 and the highest amount is found in the natural cocoa sample. The alkalized cocoa sample has one third the amount of flavonoids of the natural cocoa sample. The most important antioxidant in cocoa is (-)-epicatechin and it is not a very stable compound. (-)- Epicatechin can easily be decomposed and converted to other molecules, which do not have the same phenolic properties. This is one of the differences between alkalized and natural cocoa powders. Alkalized cocoa and natural cocoa cakes also have the same polyphenol content difference. According to Fernanda et al. (2009) cocoa polyphenols in chocolates should

have flavonoids to polyphenols ratio of around 35. Polyphenols in chocolates come directly from cocoa content and this ratio is expected to be similar for cocoa powders. Because alkalized cocoa is used in chocolate production in the food industry, alkalized cocoa's flavonoids/polyphenols ratio should be in similar range. While GAE TPC content of alkalized cocoa is close to 1800 mg/100 g cocoa, TPC in terms of QE is around 900 mg/100 g cocoa. The ratio is around 50 and this is quite reasonable when compared with Fernanda's study. The flavonoid content of cocoa cake samples is higher in natural cocoa cake samples.

3.7 Determination of Trolox Equivalent Antioxidant Activity

The aim of using ABTS method is to determine the radical scavenging activity of cocoa and cocoa cake samples. Different Trolox concentrations were used as standard to scavenge ABTS radicals in solution and to obtain a linear standard curve. Then, the inhibition percentage was calculated using the formula below:

$$\text{Inhibition } \% = (1 - A/A0) \times 100$$

A_0 was the absorbance of the control (Blank)

A_1 was the absorbance in the presence of the sample

Table 0-12 Trolox Equivalence versus Percentage of antioxidant inhibition

Final µM Trolox Eq.	% Inhibition
20.0	84.0
10.0	41.0
5.0	22.0
4.0	18.0
2.0	9.0

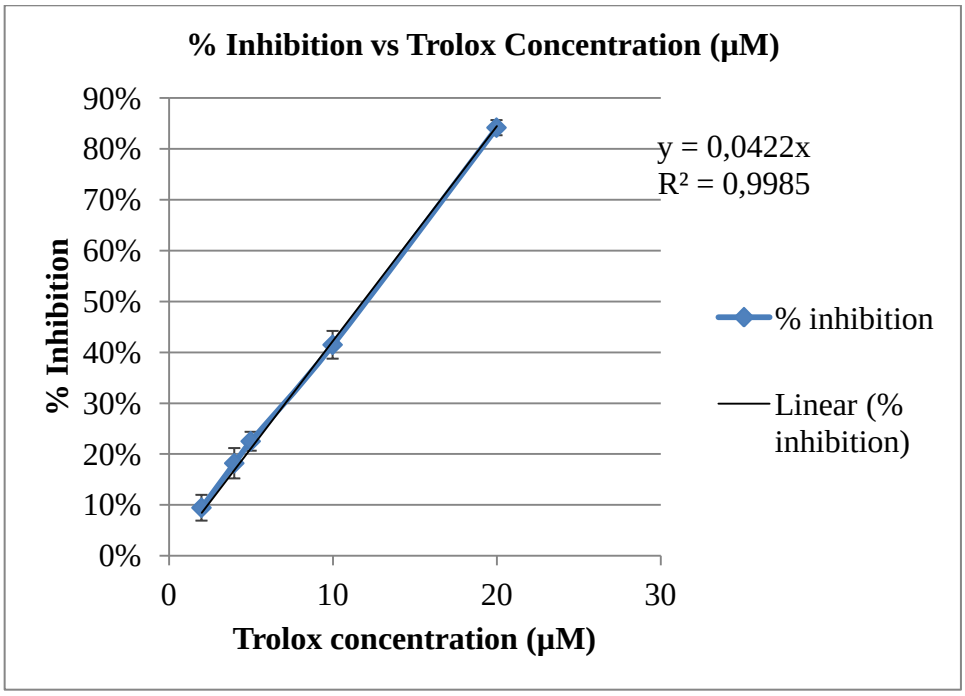


Figure 0-9 Trolox Standard curve for ABTS assay

As represented in Figure 3-9 the linear line equation y = 0.0422x gives the relation between µM Trolox concentration and inhibition percentage.

All samples were measured at least 4 times. Inhibition percentage values were calculated. In parallel to the DPPH assay results, the natural cocoa has the highest Trolox equivalent at 188.51 μmol TE/ g cocoa. Alkalized cocoa follows natural cocoa with 71.84 μmol TE/ g sample and then NCC1 with 13.35 μmol TE/ g cocoa, NCC2 with 9.89, ACC2 with 7.92 and finally ACC1 with 5.64 μmol TE/ g cake, respectively.

Table 0-13 Trolox Equivalence of Cocoa and Cocoa Cake Samples

Sample	**μmol TE/ g cocoa or cake**	**Sample**	**μmol TE/ g cocoa or cake**
AC	71.84±2.99	**NC**	188.51±4.19
ACC1	5.64±0.35	**NCC1**	13.35±0.48
ACC2	7.92±0.44	**NCC2**	9.89±0.33

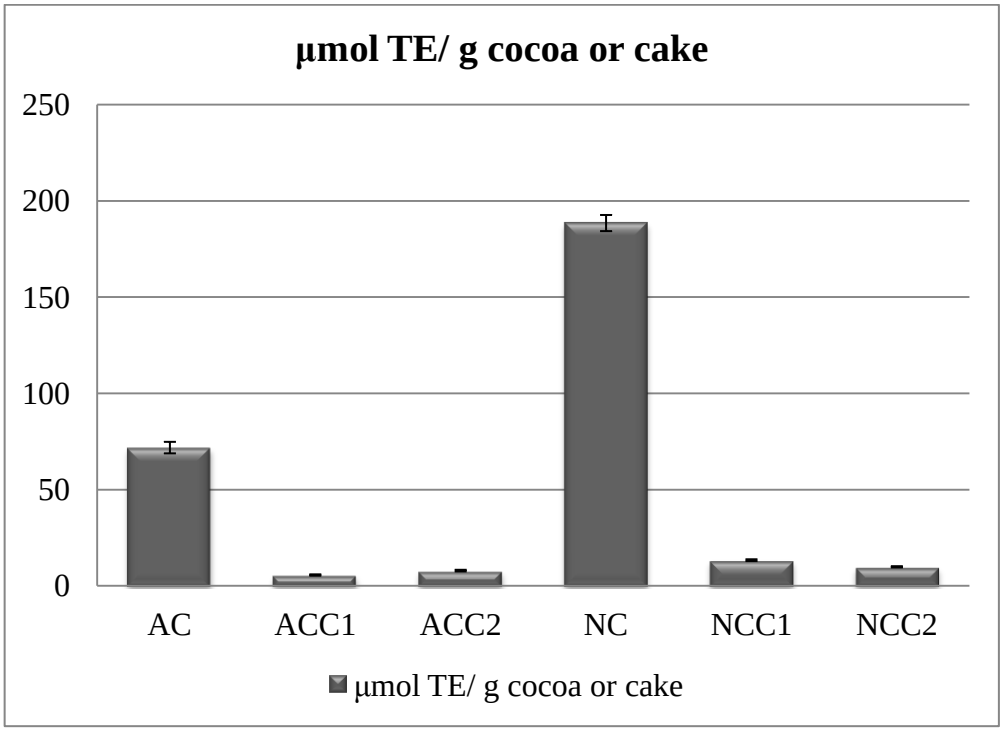


Figure 0-10 Trolox Equivalence per g sample

As represented in Figure 3-10 the antioxidant efficiency of NC is almost 2.5 times higher than that of AC. On the other hand, there is not a significant difference between the antioxidant activity of alkalized cocoa cakes and natural cocoa cakes. The results are very close to each other. In addition, the TE values of natural samples were two times higher than the alkalized samples in terms of antioxidant capacity. This result is also parallel to the results of cocoa cake samples.

According to Gu, House, Wu, Ou, & Prior (2006), TE values of natural cocoa are around 820 µmol TE/ g sample for natural cocoa powder and around 400 µmol TE/ g cocoa for alkalized (dutched) cocoa powder.

3.8 Analysis of Phenolic compounds by HPLC

High performance liquid chromatography (HPLC) was used in order to detect the (-)-epicatechin and (+)-catechin content of natural and alkalized cocoa and their cake samples.

Catechin and epicatechin standard solutions in different concentrations were prepared and injected 20µl into the HPLC device. Each standard was measured four times to obtain a certain smooth linear standard curve. Both (-)-epicatechin standard and (+)-catechin standards have different retention times. According to HPLC conditions, which were mentioned in Appendix, the retention timing was 23.62 min for (-)- Epicatechin and 15.57 min. for (+)-Catechin. The clear peaks are shown in Figure 3-11 and Figure 3-12.

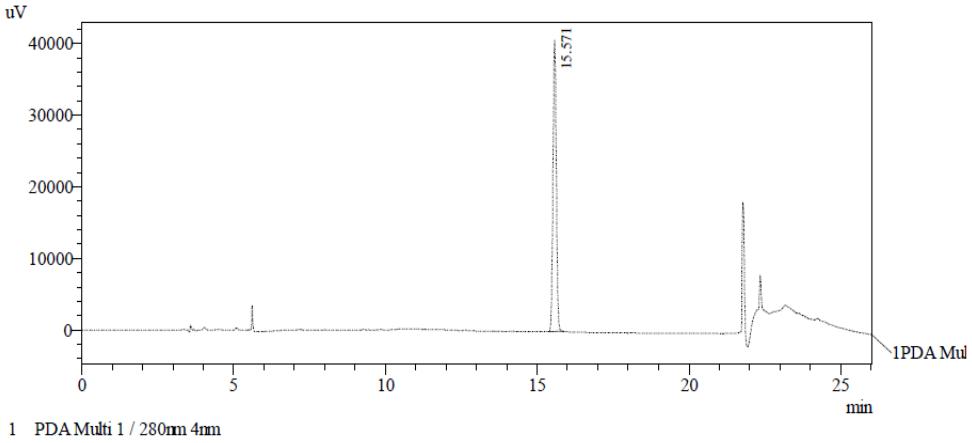


Figure 0-11 HPLC graph of Catechin Standard with retention time

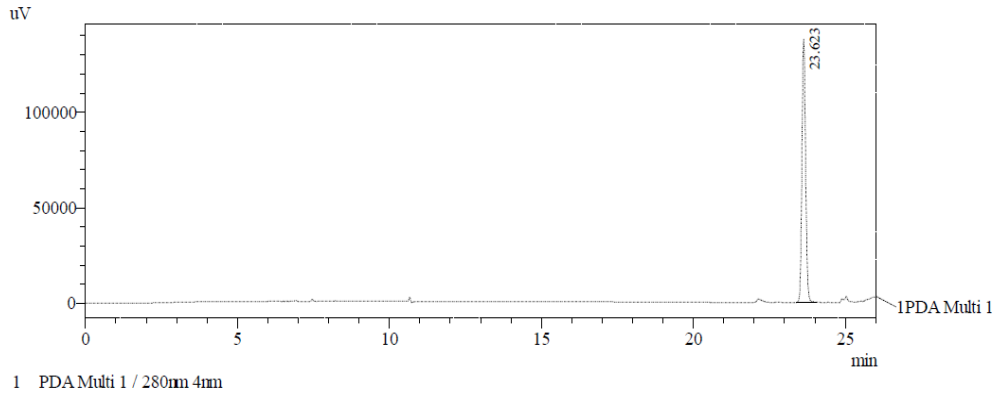


Figure 0-12 HPLC graph of (-)-Epicatechin Standard with retention time

According to area vs. concentration, the standard curves were calculated for both standards. For the (-)-epicatechin $y = 0.0000423x$ with $R^2 = 0.99$ and and $y = 0.0000785x$ for (+)-catechin. These standard curves are used to calculate the epicatechin and catechin content of the samples.

Each sample was measured by reverse phase HPLC and this process was repeated four times.

At the end of the measurements, standard curve equations were obtained.

Table 3.8 (-)-Epicatechin and (+)-Catechin results for standard curves

Epicatechin		**Catechin**	
(µg/ml)	**Ave. Area**	**(µg/ml)**	**Ave. Area**
25	570239±3395	6.25	72861±224
50	1163462±2548	12.5	146665±1326
75	1765618±6629	25	306096±2772
100	2369082±2860	50	646120±247
125	2965666±21586		

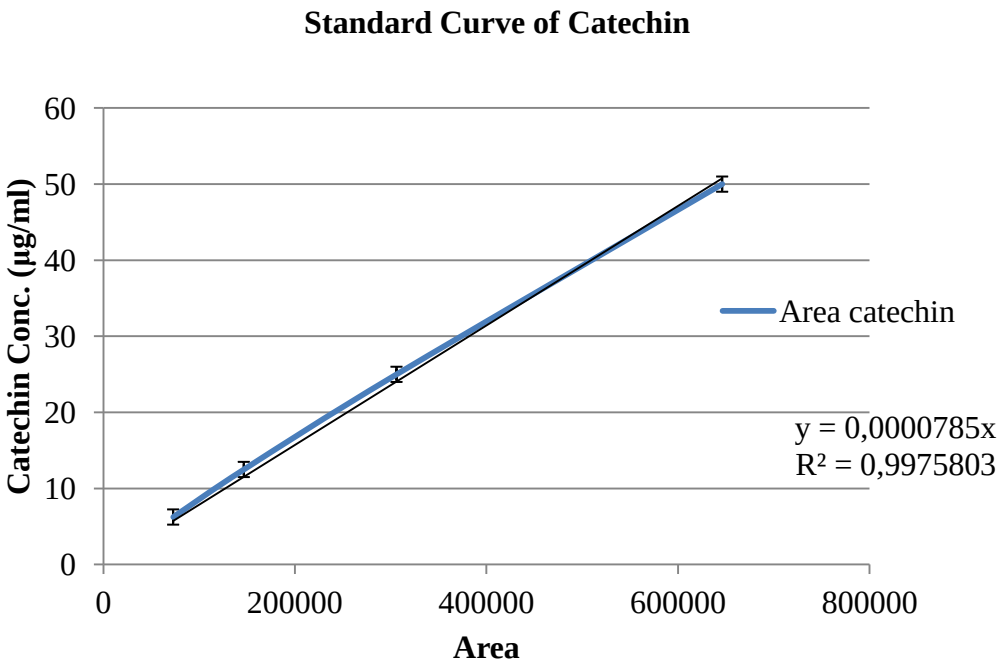


Figure 0-13 (+)- Catechin Standard Curve

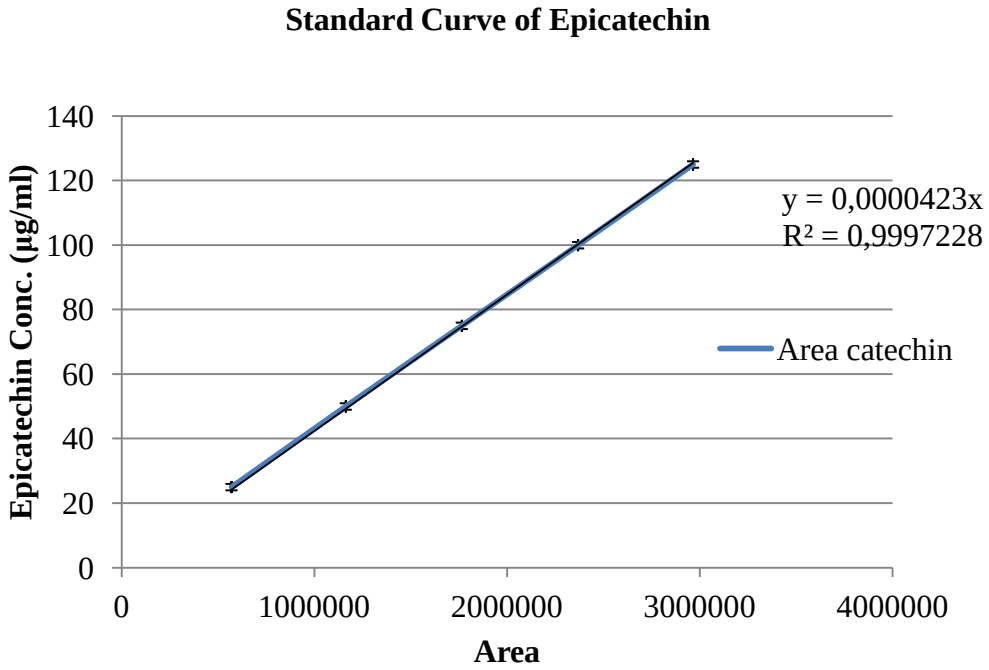


Figure 0-14 (-)- Epicatechin Standard Curve

Standard curves provide the epicatechin and catechin content of samples to be calculates as indicated below in Table 3.14.

Table 0-14 (-)-Epicatechin and (+)-catechin amount of cocoa and cake products

Sample	mg (-)-Epicatechin/100g	mg (+)-Catechin/100g	mg (-)-Epicatechin/g	mg (+)-Catechin/g
AC	17.151	32.732	0.172	0.327
ACC1	0.954	1.990	0.010	0.020
ACC2	1.209	2.266	0.012	0.023
NC	93.581	15.068	0.936	0.151
NCC1	6.868	1.037	0.069	0.010
NCC2	6.558	1.057	0.066	0.011

According to the standard curves, the real amounts of EC and C of the cocoa and cake samples were obtained by the graphs and the area under those graphs.

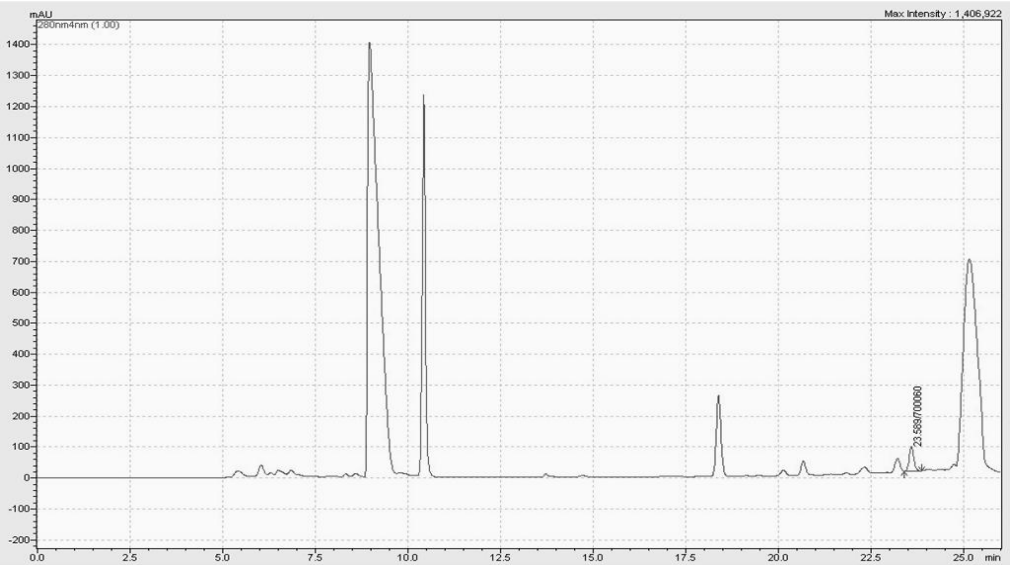


Figure 0-15 (-)- Epicatechin graph for natural cocoa sample

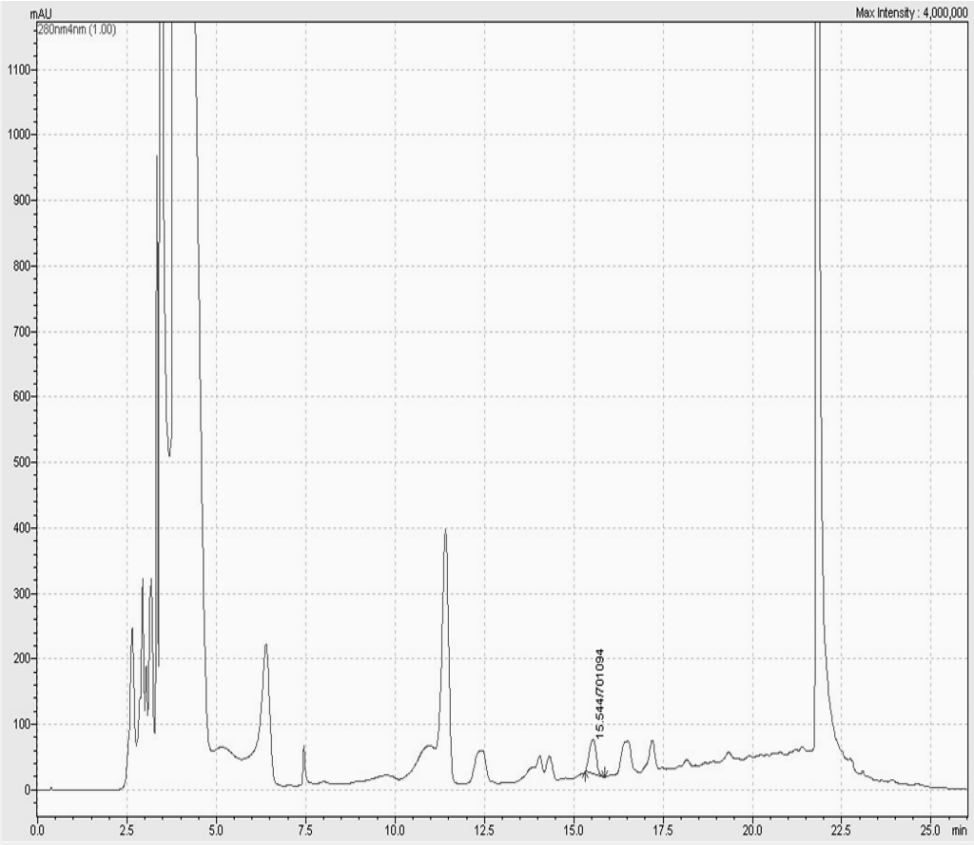


Figure 0-16 (+)-Catechin graph for alkali cocoa sample

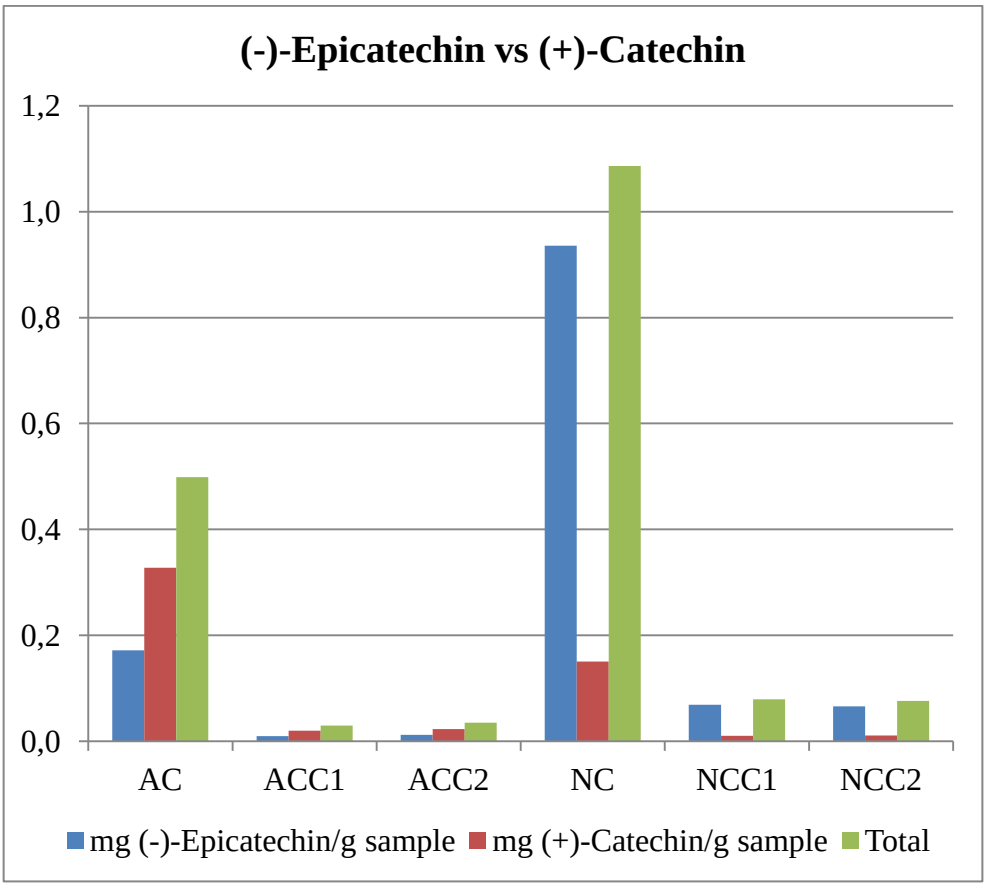


Figure 0-17 Comparison of (-)-Epicatechin and (+)-Catechin and samples are either cake or cocoa

As shown in Figure 3-12 natural cocoa contains a high amount of (-)-epicatechin 0.936±0.17 and alkalized cocoa contains 0.172 mg (-)-epicatechin per g cocoa sample. The (+)-catechin amount of NC and AC are 0.151 and 0.327mg/g cocoa sample, respectively. This result shows that while the epicatechin content decreases by processing, the catechin content increases. The dutching process causes the conversion of (-)-epicatechin to (+)-catechin. There is also the same effect in cocoa cake samples; however the total amount

of catechin and the epicatechin content is around 10% of the cocoa products, which is expected and parallel to the other analytical results.

According to Miller et al. (2009) the (-)-epicatechin content of cocoa powder sample is 1.0-2.6 mg/g cocoa and the (-)-catechin content is 0.5-0.6 mg/g cocoa. In another study the alkalized cocoa and dutched cocoa samples were compared and the EC content is around 1.5 mg/g cocoa and the C content is around 0.6 mg/g cocoa. On the other hand, for the alkalized cocoa sample, the EC content is around 0.4 mg/g cocoa and the C content is around 0.2 mg/g cocoa (Stahl et al., 2009). In addition EC content of the homemade chocolate cake sample is around 0.17-0.19 mg/g cake while the C content is around 0.06-0.08 mg/g cake.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

Cocoa samples obtained from Industrial sources, which has high antioxidant content and significant health benefits, has been studied in this research. Natural cocoa, alkalized cocoa and their cake products were the samples.

According to analytical and chromatographically analysis, natural samples significantly have the highest amount of TPC, TFC, TE and EC content. There is generally twice the difference between alkalized and natural cocoa. The cake samples generally have one to ten times lesser amounts of antioxidants. This amount was also measured with DPPH antioxidant activity measurement. The required amount of the same DPPH free radical inhibitory effect is provided with the least amount of natural cocoa and the greatest amount of alkalized cocoa cake sample. In addition it is obtained from HPLC analysis that some part of (-)-epicatechin is converted to (+)-catechin during the process. The only difference between these samples is the dutching step. This difference indicates that alkalization may cause a great decrease in the antioxidant content and activity of cocoa products.

The ratio between powders and cakes is similar. While natural cocoa has double the TPC or TFC, natural cocoa cake also doubles TPC and TFC content. However the catechin and epicatechin content differs and is parallel with the cocoa powder samples ratio. This result indicated that the heat treatment during the baking stage does not cause any decrease in the antioxidant quality and antioxidant level of the product.

This study shows that consuming natural cocoa contributes significantly higher antioxidant consumption than consuming alkalized cocoa. The bitter taste, lighter cocoa and lower pH value are the important reasons for the non-usability of natural cocoa powders in industry.

It may be recommended to research ways of improving the organoleptic properties of natural cocoa powder in food factories. If the desired properties can be achieved without lowering the antioxidants then the health benefits and impacts may be increased by consuming the same amount of cocoa containing products. It also enables the production of functional antioxidant rich healthy foods with the same taste. The results obtained in this study may lead to possible further research and clinical studies such as the effects of cocoa on human blood pressure or cardiovascular health can be organized.

The results of this study indicate that there is significant difference between natural and alkalized cocoa samples and improving the organoleptic properties of natural cocoa products could be an important strategy for the development of functional foods with significant health claims.

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APPENDIX

Table A 1 DPPH RSA% Results of Alkalized Cocoa

Final concentration (mg/ml)	RSA % of AC	Std
0.000	0.000	0.000
0.010	11.741	1.316
0.020	22.503	1.001
0.040	42.884	1.422
0.080	77.487	1.567
0.120	92.490	0.617
0.160	93.076	0.512

Table A 2 DPPH Results of Natural Cocoa

Final concentration (mg/ml)	RSA% of NC	Std
0.000	0.000	0.000
0.010	19.908	1.201
0.025	48.458	2.104
0.050	81.232	0.821
0.099	96.462	0.635
0.149	96.996	0.346

Table A 3 DPPH Results of Alkalized Cocoa Cake 1

Final concentration (mg/ml)	RSA% of ACC1	Std
0.000	0.000	0.000
0.391	10.978	1.925
0.782	23.262	0.991
1.564	42.584	1.628
2.085	54.682	2.337
3.128	74.306	1.483
4.073	81.357	1.081
5.275	84.165	1.030
7.876	85.854	1.240

Table A 4 DPPH Results of Alkalized Cocoa Cake 2

Final concentration (mg/ml)	RSA% of ACC2	Std
0.000	0.000	0.000
0.403	12.557	1.298
0.805	21.677	1.683
1.611	43.522	2.048
2.416	58.849	1.794
3.221	70.179	3.334
4.077	78.573	2.058
5.476	84.490	1.787
7.949	86.253	2.759

Table A 5 DPPH Results of Natural Cocoa Cake 1

Final concentration (mg/ml)	RSA% of NCC1	Std
0.000	0.000	0.000
0.256	17.218	0.723
0.512	29.998	0.293
0.769	41.825	0.212
1.537	73.484	1.478
2.050	88.185	1.137
3.074	92.951	0.628
4.148	93.758	1.098

Table A 6 DPPH Results of Natural Cocoa Cake 2

Final concentration (mg/ml)	RSA% of NCC2	Std
0.000	0.000	0.000
0.263	16.029	2.131
0.789	40.028	0.928
1.577	69.622	2.432
2.103	83.162	1.440
3.154	92.093	0.988
4.638	94.014	0.967

Table A 7 Absorbance for Gallic Acid Standard Curve at 750 nm

Initial Conc. mg/ml	Final Conc. mg/ml	ABS	STD
0.000	0.000	0.000	0.000
0.050	0.002	0.107	0.003
0.100	0.005	0.241	0.008
0.200	0.009	0.514	0.012
0.300	0.014	0.763	0.026
0.400	0.018	1.087	0.073

Table A 8 Absorbance for Quercetin Standard Curve at 510 nm

Initial conc. mg/ml	Final conc. mg/ml	ABS Ave	Std Dev
0.000	0.000	0.000	0.000
0.100	0.010	0.136	0.021
0.200	0.020	0.325	0.016
0.300	0.030	0.487	0.011
0.400	0.040	0.677	0.006
0.500	0.050	0.859	0.012

Table A 9 HPLC Conditions

Data Acquisition	
LC Stop Time	26 min
Acquisition Time (PDA)	1.5625 Hz
Start Time	0 min
End Time	26 min
Time Constant	0.630 sec

Table A 10 HPLC Conditions-Oven

Column Oven	
Model	CTO-20A
Oven Temperature	37 ℃
Maximum Temperature	85 ℃

Table A 11 HPLC Conditions- LC

LC Time (min)		
0.01	B	6
6	B	6
18	B	25
19	B	60
21	B	60
23	B	25
25	B	6
26	STOP	

Table A 12 HPLC Conditions -Pump

Pump	
Mode	Lower pressure gradient
Total Pump Flow	0.5 ml/min
Solvent B Con	6%
Solvent A Con	94%
Maximum Pressure	20 MPa
Minimum Pressure	0 Mpa

Table A 13 HPLC Conditions-PDA

PDA	
Model	SPD-M20A
Lamp	D2&W
Start Wavelength	190 nm
End Wavelength	800nm
Cell Temperature	40 ℃
Slit Width	1.2nm

Table A 14 HPLC Conditions - Autosampler

Autosampler	
Model	SIL-20A
Autosampler	
Sample Rack	Rack 1.5ml 105 vials
Rinsing Volume	200υl
Needle Stroke	52 mm
Control Vial Need Stroke	52 mm
Rinsing Speed	35υl/sec
Sampling Speed	15υl/sec
Purge Time	25 min
Rinse Mode	Before and After aspiration