INVESTIGATION OF ANTIOXIDANT CAPACITY AND PHENOLIC CONTENTS OF CHOCOLATES IN THE TURKISH MARKET

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

BAŞAK ÖVET

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
FOOD ENGINEERING

MAY 2015

Approval of the thesis:

INVESTIGATION OF ANTIOXIDANT CAPACITY AND PHENOLIC CONTENTS OF CHOCOLATES IN THE TURKISH MARKET

submitted by **BAŞAK ÖVET** in partial fulfillment of the requirements for the degree of **Master of Science in Food Engineering Department, Middle East Technical University** by,

Prof. Dr. Gülbin Dural Ünver _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Alev Bayındırlı _____
Head of Department, **Food Engineering**

Prof. Dr. Faruk Bozoğlu _____
Supervisor, **Food Engineering Dept., METU**

Assoc. Prof. Dr. Gülçin Sağdıçoğlu Celep _____
Co-supervisor, **Family and Consumer Sciences Dept., Gazi University**

Examining Committee Members:

Prof. Dr. Musa Doğan _____
Biology Dept., METU

Prof. Dr. Faruk Bozoğlu _____
Food Engineering Dept., METU

Assoc. Prof. Dr. Gülçin Sağdıçoğlu Celep _____
Family and Consumer Sciences Dept., Gazi University

Assoc. Prof. Dr. Ahmet Emre Yaprak _____
Biology Dept., Ankara University

Assist. Prof. Dr. Yeşim Soyer _____
Food Engineering Dept., METU

Date: 06.05.2015

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Başak Övet

Signature:

ABSTRACT

INVESTIGATION OF ANTIOXIDANT CAPACITY AND PHENOLIC CONTENTS OF CHOCOLATES IN THE TURKISH MARKET

Övet, Başak

M.S., Department of Food Engineering

Supervisor: Prof. Dr. Faruk Bozoğlu

Co-Supervisor: Assoc. Prof. Dr. Gülçin Sağdıçoğlu Celep

May 2015, 100 pages

Consumption of polyphenol rich foods is highly associated with human health, mainly with cardiovascular homeostasis. Chocolate is a very rich source of flavonoids, mainly (-)-epicatechin (EC), which has several reported bioactivities. Beneficial effects of polyphenols are usually attributed to their antioxidant potential. Therefore in this study, different chocolate samples commonly found in Turkish market were studied for their antioxidant capacities using $DPPH^{\bullet}$ and $ABTS^{\bullet}$ radicals scavenging methods. Total phenolic and flavonoid contents were determined. EC and (+)-catechin (C) contents of the samples were investigated using RP-HPLC.

For $DPPH^{\bullet}$ radical scavenging activities, IC_{50} values were determined for the milk and dark chocolate samples as 2.652±0.093 mg extract/ml and 0.473±0.07 mg extract/ml, respectively. For $ABTS^{\bullet}$ radical scavenging activities, TE values were determined for the milk and dark chocolate

samples as 29.21±2.57 μmol TE/g and 144.09±16.56 μmol TE/g chocolate, respectively. The total phenolic contents were determined as 282.48±15.74 mg GAE/100 g chocolate for milk and 1010.24±76.68 mg GAE/100 g chocolate for dark chocolate samples. The total flavonoid contents were determined as 168.67±9.22 mg/100g milk chocolate and 568.81±48.54 mg/100 g dark chocolate.

The EC content for milk chocolates were determined as 0.069±0.050 mg EC/g chocolate and for dark chocolates 0.192±0.081 mg EC/g chocolate. The C content of the milk chocolate was determined as 0.090±0.033 mg C/g and for dark chocolate samples as 0.221±0.040 mg C/g chocolate. Antioxidant capacities, total phenolic, total flavonoid and EC contents of dark chocolate samples were significantly higher than milk chocolate samples.

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

ÖZ

TÜRKİYE PİYASASINDA BULUNAN ÇİKOLATALARIN ANTİOKSİDAN KAPASİTELERİ VE FENOLİK MADDE İÇERİKLERİ YÖNÜNDEN ARAŞTIRILMASI

Övet, Başak
Yüksek Lisans, Gıda Mühendisliği Bölümü
Tez Yöneticisi: Prof. Dr. Faruk Bozoğlu
Ortak Tez Yöneticisi: Doç. Dr. Gülçin Sağdıçoğlu Celep

Mayıs 2015, 100 sayfa

Polifenol içeriği zengin gıdaların tüketimi, insan sağlığı ile özellikle de kardiyovasküler sağlık ile ilişkilendirilmektedir. Çikolata, flavonoidlerin özellikle de çok çeşitli biyoaktiviteleri gösterilmiş olan (-)-Epikatekin'in (EC) iyi bir kaynağıdır. Polifenollaerin sağlık üzerindeki olumlu etkileri genellikle antioksidan potansiyallerine bağlanmaktadır. Bu nedenle bu çalışmada, Türkiye pazarlarında sıklıkla bulunan farklı çikolata türlerinin antioksidan kapasiteleri, $DPPH^{\bullet}$ ve $ABTS^{\bullet}$ radikallerini yakalama yöntemleri ile ölçülmüştür. Örneklerin toplam fenolik ve flavonoid madde içerikleri tayin edilmiştir. Örneklerde bulunan EC ve (+)-Katekin (C) miktarları RP-HPLC yöntemi ile tayin edilmiştir.

Bitter çikolata örneklerinin DPPH radikali yakalama kapasiteleri 0.473±0.07mg/ml, sütlü çikolata örneklerinin DPPH radikali yakalama kapasiteleri ise 2.652±0.093 mg/ml olarak tayin edilmiştir.

ABTS$^{\bullet}$ radikali yakalama kapasiteleri için TE değerleri, bitter çikolata örneklerinin 144.09±16.56 μmol TE/g çikolata, sütlü çikolata örneklerinin ise 29.21±2.57 μmol TE/g çikolata olarak tayin edilmiştir. Bitter çikolata örneklerinin toplam fenolik içerikleri 1010.24±76.68 mg GAE/100 g çikolata ve sütlü çikolata örneklerinin toplam fenolik içerikleri 282.48±15.74 mg GAE/100 g çikolata olarak ölçülmüştür

Toplam flavonoid içerikleri, bitter çikolata örneklerinin 568.81±48.54 mg/100 g ekivalan çikolata, sütlü çikolata örneklerinin ise 168.67±9.22 mg / 100 g ekivalan çikolata olarak ölçülmüştür.

Sütlü çikolata örneklerinin EC miktarları 0.069±0.050 mg EC/g örnek çikolata, bitter çikolata örneklerinin ise 0.192±0.081 mg EC/g çikolata olarak belirlenmiştir. Sütlü çikolata örneklerinin C miktarları 0.090±0.033 mg C/g, bitter çikolata için ise 0.221±0.040 mg C/g çikolata olarak belirlenmiştir.

Bitter çikolata örneklerinin antioksidan kapasitenin, toplam fenolik, toplam ve EC miktarlarının, sütlü çikolataya göre önemli ölçüde daha yüksek olduğu saptanmıştır.

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

Dedicated to my precious parents…

ACKNOWLEDGEMENTS

During these studies, I always felt all the support of my family, advisors and friends. I am mostly grateful to my beloved parents Gülser Zehra & Süleyman Övet. Throughout the whole of my life I always feel their infinite support and invaluable love. Nothing is possible without their support. I also would like to thank to my beloved sister Banu Özkan and my precious nephew Demir Özkan for their all love and support. My achievements follow my sister's achievements' footprints.

I would like to thank my supervisor Prof. Dr Faruk Bozoğlu and my co-advisor Assoc. Prof. Dr A. Gülçin Sağdıçoğlu Celep. They always encouraged and supported me during this study. Then, I would like to thank to Research Asst. Ahmet Altay for his guidance during the experiments and during this study.

I would like to send my special thanks to Assist. Prof. Dr. Nilüfer Didiş and Assist. Prof. Dr. Gözde Didiş for their support and technical advices during the preparation of this study. I would like to thank to my friends Meltem Karadeniz and Elçin Bilgin for all their friendship. In addition, my managers and my dear friends that I gained in Eti Company also gave me their continuous friendship and support during my working and studying together period. I would also like to thank to my friends and managers in Mondelēz International for their encouragement and support.

Lastly, I am totally thankful to Mert Cem Didiş because of all his beloved endless love, patience and support. Without his support and love this target could not be achieved.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

CHAPTER 1

INTRODUCTION

In recent years, the awareness of consumers about the impact of diet on human health considerably increased. Particularly high polyphenol containing diet gained importance due to its beneficial effects on cardiovascular health and vascular homeostasis. Polyphenols are one of the most important dietary antioxidants and flavonoids are a subgroup of them.

Cocoa is one of the richest sources of flavonoids, mainly flavan-3-ols and it is the main ingredient of chocolate. Therefore chocolate, particularly dark chocolate also contains high amounts of flavonoids. (-)-Epicatechin (EC) is one of the most studied bioactive compounds of cocoa and chocolate regarding to its antioxidant activity. It can effectively scavenge free radicals and prevent oxidative damage in tissues.

Several studies have reported the importance of dark chocolate consumption on cardiovascular health. Besides their free radical scavenging activities, these bioactive compounds are denoted to be effective in prevention of several important degenerative diseases such as Alzheimer, cancer, aging, and high blood pressure.

In this study, different milk and dark chocolates commonly found in Turkish market were selected in order to investigate their antioxidant activities by using different analytical and chromotographical methods. The main active ingredient of chocolate (-)-Epicatechin (EC) and (+)-Catechin (C) contents of the samples were also investigated analytically by using RP-HPLC.

1.1 Chocolate

Chocolate, a widespread consumed snack, is one of the most popular food in worldwide and its existence comes from centuries. Cocoa is the main ingredient of chocolate and chocolates are grouped depending on their cocoa contents and the presence of other ingredients. Chocolate is also used as an ingredient in the production of other foods such as cakes containing chocolate, chocolate covered ice creams, puddings with chocolate, and coffees including chocolate sauces, etc. Cocoa is derived from cocoa beans which are the seeds of the cocoa trees, *Theobroma cacao* mainly planted in Ecuador, Brazil, Central and South America, and West Africa (Afoakwa, 2010). Recently, the number of chocolate companies producing different types of chocolates with a wide range of portfolio has increased.

1.1.1 History of Chocolate

Chocolate has been one the most favorite foods for more than 4000 years. The first bare bones of the chocolate were constructed by The Mayan society. Some production steps which construct the origin of today’s chocolate have been coming from those ages such as fermentation, drying, roasting and grinding. (Patton, Ford, & Crunkleton, 2005).

Cocoa is the main content of the chocolate. It was named by Linnaeus in 1753 as *Theobroma cacao*, the Greek words "theo" and "broma" meaning food of

Gods (Latif, 2013). According to Afoakwa (2010) that cocoa term is derived from Aztec and Mayan languages; known as “chocolatl”.

When chocolate was first produced for consumption, it was rather a drink or a blocked mass prepared by dissolving cocoa in water or milk. This bitter drink was started to be consumed in Central and South America and in 1502, Spanish people started to add vanilla, sugar, cinnamon, honey, beer or vine (Afoakwa, 2010; Patton et al., 2005).

The first chocolate factory was founded in 1728 by British Fry family (Afoakwa, 2010) and the first chocolate in US was founded in 1778 (Afoakwa, 2010). However as a drink, some properties of chocolate were required to be improved. It was very fatty because of the high cocoa fat content in cocoa beans which made the blocked chocolate masses hard to be dissolved in hot water and fat particles were floating on the top of the drink, causing unpleasant look (Beckett, 2008).

After a while, the Dutch, Van Houten in 1828 discovered cocoa press in order to remove fat from cocoa. At those times, cocoa press method was providing half of the fat content to be removed from cocoa beans and the output was hard cocoa solids which were known as “cake” and cocoa butter. By this process better dispersion in hot water and in milk was provided and with this improvement cocoa demand increased significantly (Beckett, 2008).

After this invention, an eating chocolate was first discovered by Joseph Fry in 1847 in Bristol in the UK by mixing the chocolate masses with milled sugar (Afoakwa, 2010; Patton et al., 2005). However those chocolate blocks were very crumbling. At the same time, for the cocoa cake producers, it was necessary to find a market in which they needed to sell their cocoa butter. Both were provided at the same consequence; additional cocoa butter in the roasted cocoa nib and milled sugar mix (Beckett, 2008). Mixing of additional cocoa butter supplied a hard and uniform texture to the chocolate and this

improvement made its consumption and appearance more convenient. With these properties, chocolate became one of the most unique foods in the world, with its smooth and hard texture under normal conditions and melting with body temperature.

After all these inventions, first milk chocolate was created by the addition of milk and extra sugar to those bars made by Fry. The inventors of the first milk chocolate were Henri Nestle and Daniel Peters and they started to make it in 1894 (Patton et al., 2005). Afterwards, many other companies such as Cadbury, Rowntree and Hershey's were constructed to produce chocolate (Beckett, 2008).

1.1.2 Types and Production of Chocolate

1.1.2.1 Types of chocolate

Chocolates in plain format are grouped in three different types according to their cocoa contents and additional ingredients. White chocolate, milk chocolate and dark chocolate are the main groups of chocolates and their cocoa solid content is regarded as lowest, medium and highest, accordingly. Table 1.1 presents the chocolate types and their cocoa contents together with their visuals.

Table 1.1 Types of chocolate.

Type of chocolate	Cocoa Content	Visuals
White	Only cocoa butter / no cocoa powder	
Milk	10% chocolate liquor & additional cocoa butter	
Dark	40% chocolate liquor & very little added cocoa butter	

Table is adapted from Patton et al. (2005).

Recently chocolates are used as the key ingredients in several different processes and products for various different purposes in food industry. For instance; coating of the ice creams, cakes or biscuits, making different shaped bars with additional other ingredients, making truffles, making birthday cakes, coating fruits, making fondue, etc.

Consumers should be aware of the labeling of chocolates. Some products cannot be regarded as real chocolate. These products are named cocoa coating, cocolin, or praline. The main criterion in order to name a chocolate as "chocolate" is its cocoa content. The imitation chocolates are made up from vegetable oils, and do not contain cocoa butter. It can be advantages in terms of certain process conditions. For instance, the imitation chocolates do not melt easily, or do not have fat bloom problem as much as the real chocolates. However the taste and the mouth feeling quality are much higher in real chocolates.

In addition to those three chocolate types, there are also other chocolates with higher cocoa content than general dark chocolate. Lindt, one of the most popular chocolate brands in USA, has high cocoa containing dark chocolates. They have 70, 85, 90 and even 99% cocoa content in their special products. However in Turkey and in most of the other countries having different chocolate brands, many dark chocolates have maximum cocoa content around 50%.

According to Turkish Food Codex Regulations about Chocolate and Chocolate Products which is published in 2003, chocolate types classification according to their cocoa dry solid content is explained in Table 1.2 with the information giving the lower limits of cocoa solid content, and milk solid content.

Table 1.2 Chocolate types which are grouped according to their cocoa dry solid content. (Turkish Food Codex Regulation, 2003)

Chocolate Type	**Cocoa Fat Content (%)**	**Cocoa Non Fat Solid Content (%)**	**Total Cocoa Solid Content (%)**	**Milk Fat Content (%)**	**Milk Solid Content (%)**	**Cocoa Fat And Milk Fat Content (%)**
Dark Chocolate	18	14	35	-	-	-
Milk Chocolate	-	2.5	25	3.5	14	25
White Chocolate	20	-	-	3.5	14	-

These values are minimum required amounts in terms of percentages in a chocolate which is legally approved to be sold as a chocolate by the Turkish Food Codex. If a chocolate does not comply with these percentages, it is not allowed to be sold as a dark chocolate, milk chocolate, or white chocolate.

In Table 1.1 the cocoa solid content percentages are the lowest allowed limits and the most trustful chocolate brands in Turkey complies with these limits. Moreover, they put additional amounts of cocoa solid content in order to reach higher quality. In recent years, famous chocolate companies are involved in research and development studies to produce high quality chocolates with high levels of cocoa and milk contents. Some companies produce around 70% or 80% cocoa containing dark chocolate because the palatability of the chocolate increases with cocoa content, so the main characteristic taste of chocolate is felt due to the presence of intense cocoa. On the other hand, increased milk solid content around 30-35% of the milk chocolate provides better milky taste to chocolates.

Complying with these regulations and adding more cocoa solid content to the chocolate provides more health benefits due to the antioxidants coming naturally with cocoa content. However in Turkish Food Codex, there is no special term or definition indicating specially the chocolates with the high levels of cocoa contents such as 80% or 90%.

1.1.2.2 Production process of chocolate

Main ingredient of the chocolate is cocoa therefore cocoa production is the first step in chocolate production process. However there are not too many chocolate production companies which are producing their own cocoa beans. The main reason behind this is that cocoa is a very special plant which can grow only under certain circumstances and around Ecuador region. Cocoa beans require fermentation process immediately after being collected from the

trees. Otherwise, if cocoa pods are transported directly without fermentation, they will have a high risk of deterioration. It is also very expensive in terms of transportation after they are collected (Beckett, 2008, p.8). Consequently, instead of growing their cocoa trees, chocolate producers prefer to obtain fermented and dried cocoa beans to produce their own cocoa products. In the production of cocoa powder, the following processes for dried beans are cleaning, shelling, winnowing, alkalizing and roasting. Cleaning, shelling and winnowing steps provide the removal of cocoa shells. Clean cocoa beans are obtained when the shells are removed totally during the winnowing period (Zoumas, Azzara, Bouzas, 2000). Alkalizing is optional, and it is possible to produce cocoa powders without alkalization. Non-alkalinized cocoa powders are classified as natural cocoa powders. In the chocolate production process, alkalization step is applied in most of the cases, because it reduces the bitter taste of cocoa in the final product. The roasting step is very critical, because it gives the most characteristic taste to the final product. Chocolate liquor, the most important characteristic part of the chocolate for taste, texture and quality, is added into the chocolate directly. It is produced by grinding and refining of the fermented and dried cocoa beans. The following steps are shown on the Figure1.1 (Patton et al., 2005).

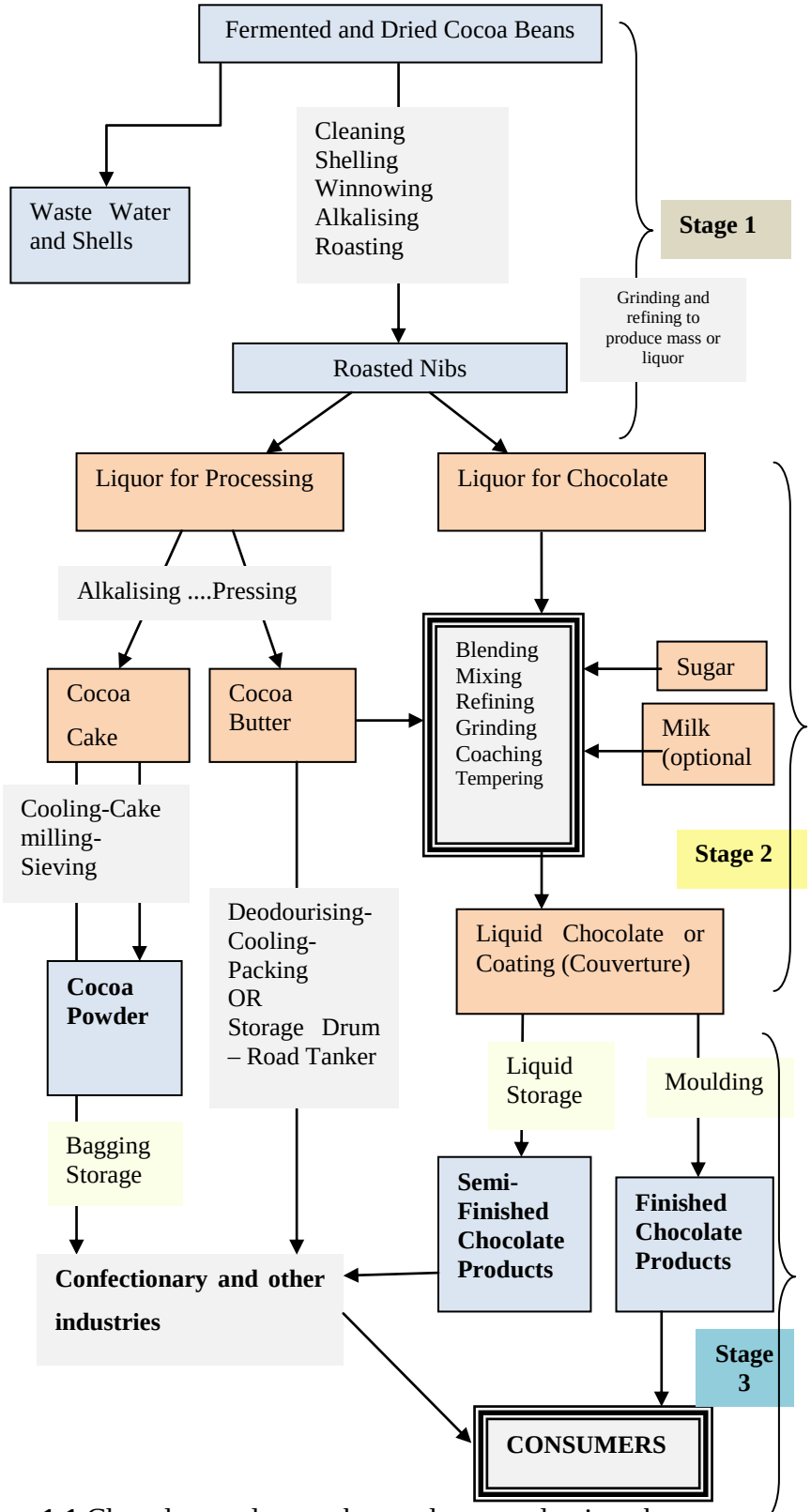


Figure 1.1 Chocolate and cocoa by products production chart

1.1.3 Ingredients and Nutrition Facts of Chocolate

Chocolate is usually consumed as a snack which has less complex ingredient content among other snacks such as crackers, cakes, and candies. Type, desired taste or functionality are the indicators of the ingredients of chocolate, therefore ingredients list of chocolates should be examined for all different types.

White chocolate: Ingredients used to produce a white chocolate may differ for each different brand. The main ingredients are sugar, milk solids, cocoa butter, soy lecithin, and vanillin. In some cases, skimmed milk powder, whey powder or lactose can also be used. According to European Council in 2000, in order to be able to label a chocolate as a white chocolate, it is a must to add whole milk powder and cocoa butter. Otherwise, if the cocoa butter is replaced by another fat, then it is not possible to name it as white chocolate and instead, it should be named as a chocolate substitute. During white chocolate manufacturing, sugar and milk particles are covered by cocoa butter fatty phase. Table 1.3 presents the ingredients of white chocolate (Vercet, 2002).

Table 1.3 An example of ingredients list for white chocolate and white chocolate substitute. (Vercet, 2002)

Ingredients	White chocolate (%)	White chocolate substitute (%)
Sugar	53.0	50.0
Added cocoa butter	**23.0**	**-**
Cocoa butter replacer	**-**	**26.0**
Skimmed milk powder	11.0	11.0
Whole milk powder	8.6	10.0
Lactose	4.0	2.7
Lecithin	0.4	0.3

Milk chocolate: The main difference between white chocolate and milk chocolate is their cocoa powder contents. Cocoa powder or chocolate liquor is not added to white chocolate in its production however, while making milk chocolate; it is necessary to add cocoa mass such as powder or liquor to the recipe. There are some different formulations and the Table 1.4 shows the typical recipe ingredient amounts as intervals.

Table 1.4 Different example formulations in order to make a milk chocolate.

Ingredients	**Example 1 Amounts (%)**	**Example 2 Amounts (%)**	**Example 3 Amounts (%)**
Chocolate liquor	11.0	12.0	12.0
Whole milk powder	13.0	15.0	20.0
Sugar	54.6	51.0	45.0
Added cocoa butter	21.0	21.6	22.6
Lecithin	0.3	0.3	0.3
Vanillin	0.1	0.1	0.1
Total Fat	*30.9*	*32.6*	*35.0*

Table is adapted from (Zoumas et al., 2000).

Dark Chocolate: Dark chocolate, generally known and sold by the name bitter chocolate, is different both from white and milk chocolate in terms of cocoa content intensity. Milk powder is not used in dark chocolate production and it is both possible to use only chocolate liquor or chocolate liquor and cocoa butter together. Table 1.5 explains the different formulations for making a dark chocolate.

Table 1.5 Different example formulations in order to make milk chocolate

Ingredients	Example 1 Amounts (%)	Example 2 Amounts (%)	Example 3 Amounts (%)
Chocolate liquor	15.0	35.0	70.0
Sugar	60.0	50.4	29.9
Added cocoa butter	23.8	14.2	-
Lecithin	0.3	0.3	-
Vanillin	0.9	0.1	0.1
Total Fat	*32.0*	*33.0*	*37.1*

Table is adapted from (Zoumas et al., 2000)

In terms of nutrition facts, it is more accurate to classify according to the types of cchocolate as in the same case of ingredients list. (Afoakwa, 2010).

Table 1.6 Major nutritional constituent comparisons between white, milk and dark chocolate

Chocolate Type	Carbohydrate (%)	Fat (%)	Protein (%)
Dark Chocolate	63.5	28.0	5.0
Milk Chocolate	56.9	30.7	7.7
White Chocolate	58.3	30.9	8.0

Table is adapted from Afoakwa et al. (2007a, as cited in Afoakwa, 2010).

Main macronutrients vary accordingly to the chocolate types and there are also micronutrients available in chocolates. For example milk and cocoa are the sources of calcium and copper respectively. Table 1.7 presents nutrition facts of different chocolates including micronutrients.

Table 1.7 Micronutrients of different chocolates in 100 g portion chocolate

Chocolate Type	Calcium (mg)	Magnesium (mg)	Iron (mg)
White chocolate	272	27	0.2
Milk Chocolate	224	59	2
Plain Chocolate	32	90	3

Table is adapted from Beckett (2008).

According to Food Standards Agency (2002 as cited in Aisbitt, 2008) it is possible to compare the reference required intakes for macronutrient as GDA (Guideline Daily Amount) and for micronutrient contents as RDA (Recommended Dietary Allowances) with the amounts coming from 100 g chocolate.

Table 1.8 Nutrient Facts of 100 g chocolate and GDA/RDA values of those nutrients.

Nutrient	Dark Chocolate	Milk Chocolate	White Chocolate	GDA / RDA for adults
Energy (kcal)	510	520	529	2000
Fat (g)	28	30.7	30.9	70
Carbohydrate (g)	63.5	56.9	58.3	230
Protein (g)	5	7.7	8.0	45
Iron (mg)	**2.3**	1.4	0.2	14
Calcium (mg)	33	**220**	**270**	800
Magnesium (mg)	**89**	50	26	300

Table is adapted from Food Standards Agency (2002, as cited in Aisbitt, 2008)

In the literature, several different examples of the nutrition facts are given for different chocolate formulations. The nutrition facts of chocolate depend on the ratios and quality of the ingredients used. For instance, when high quality milk is used, the calcium or protein content of chocolate increases. Carbohydrate or the fat amount used in the production of chocolate also affects the bitterness of the dark chocolate. Fat content of 70% cocoa containing dark chocolate is higher than than 54% cocoa containing dark chocolate.

1.1.4 Physical Properties of Chocolate

Chocolate is one of the most unique products with its solid structure at room temperature and liquid structure in the mouth at body temperature. This structure is a result of range of the melting point of cocoa butter and crystals in the chocolate (Morgan, 1994). The tempering step of chocolate production provides desired ß5 crystals stability in the chocolate during the shelf life period. With this process chocolate reaches to a smooth and mouth coating creamy liquid structure while eating. The particle size determines the quality of chocolates and smaller particle size around 10-12 μm provides premium quality chocolates. For lower quality chocolates such as coating chocolates, the particle size can increase to 80μm (Schenker, 2000). In addition the flavor, taste and odor of chocolate depend on not only cocoa but also other ingredients (Lass, 1999).

1.2 Oxidants and Antioxidants

Species capable of existing independently with one or more unpaired electrons are called free radicals (Halliwell, 1989). These highly reactive compounds are dangerous for the cells when their cellular production exceeds the antioxidant capacity (Sagdicoglu Celep and Marotta, 2014). Even though oxygen is essential for life, it is also classified as a free radical and it can have damaging

effects on human body. Reactive Oxygen Species (ROS) can react with biological molecules and causes oxidative stress in cells and harmful reactions might occur (Craft, Kerrihard, Amarowicz, & Pegg, 2012).

ROS such as $O_2^{\bullet -}$ or $HO^{\bullet}$ have adverse effects on health when produced in high concentrations (Halliwell, 1995). There are also non-radical oxidizing agents such as hydrogen peroxide (H_2O_2), ozone (O_3) and Reactive Nitrogen Species (RNS) $NO^{\bullet}$, $NO_2^{\bullet}$. They may also cause oxidative stress in the cells (Halliwell, 1995).

ROS can be produced in the cells by a number of different chemical processes including enzymatic reactions, drugs, toxic compounds, tobacco smoke, radiation and other environmental factors (Sagdicoglu Celep and Marotta, 2014). The most important sources of free radicals produced in aerobic cells are the electron transport chains of mitochondria where approximately 1-5% of all oxygen used in metabolic processes escapes as free radicals, mainly as $O_2^{\bullet -}$ (Halliwell, 1989).

Free radicals can react with biological molecules and can induce cellular damage. They may react with sugars, amino acids, phospholipids and nucleotides and damage the integrity of cells. OH• is one of the most reactive chemical species known to be able to react with DNA and RNA. They may initiate the peroxidation reactions of lipids in the membranes or initiate DNA breakage. They play role in the pathogenesis of many diseases including atherosclerosis, neurodegenerative diseases such as Alzheimer's and Parkinson's, cancer and aging. One important result of free radical increase in the body is plaque formation in veins causing cardiovascular diseases. Free radical damage and general contribution of oxidative stress to diseases is given in Figure 1.2.

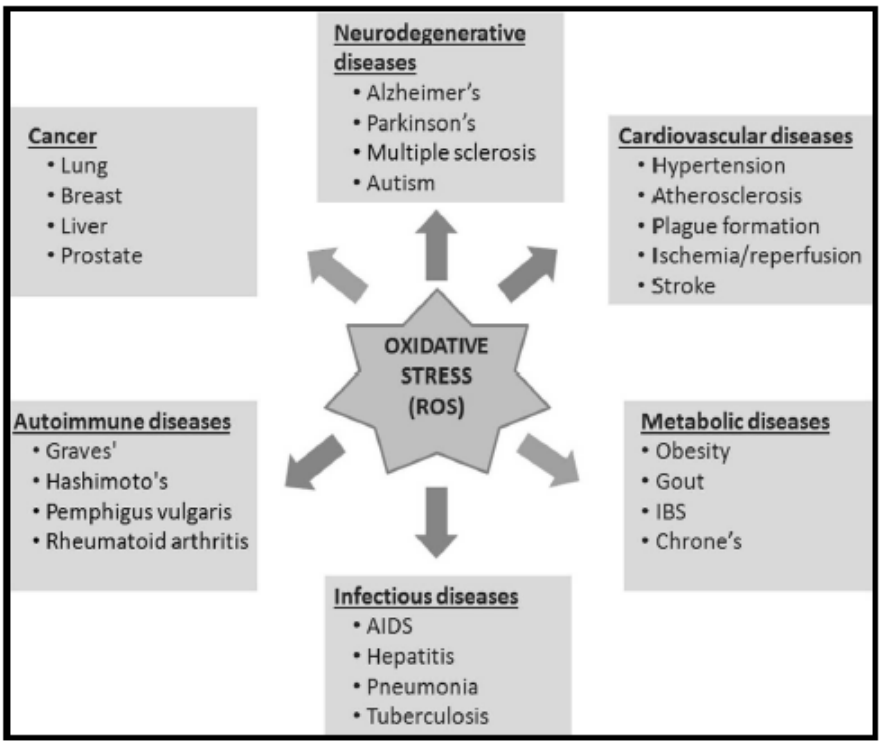


Figure 1.2 Oxidative stress and its general contribution to diseases (Sagdicoglu Celep and Marotta, 2014)

1.2.1 Mechanism of Antioxidants

Antioxidants have 2 main mechanisms to inhibit oxidative damage, which are chain breaking and free radical scavenging mechanisms. Free radicals produced nearby the lipid membranes causes lipids to be oxidized which leads to formation a group of deleterious reactions called lipid peroxidation chain reactions. Termination, initiation, and propagation steps of lipid peroxidation chain reactions are shown below (Craft et al., 2012).

$$RH + Initiator \rightarrow R\bullet + H\bullet \text{ (Initiation)}$$

$$R\bullet + O_2 \rightarrow ROO\bullet \text{ (Propagation)}$$

ROO • + R'H → ROOH + R'• (Propagation)

ROO • + R• → ROOR (Termination)

R • + R • → RR (Termination)

The initiation step is the increase of the free radical quantity, the second step which is propagation provides the increased diversity of the free radical type with the same number. The following step is the termination and in this step free radical quantity decreases.

Antioxidants are one of the most important substances which provide beneficial interactions in the human body by providing free radical scavenging activity and helping to decrease the risk of oxidation and intake of foods with high antioxidant capacity is beneficial in protecting the human body from the harmful effects of free radicals.

In food industry, prevention of foods from oxidation is a very important issue particularly for foods with high lipid content and foods stored under sunlight. This causes deterioration of the food stuffs, loses of palatability and taste of the products, and shortened shelf life. In order to protect food materials from exposed oxygen, antioxidants are commonly added as food additives.

European Union approved a list of additives with E numbers that can be used in food industry. These E numbers are used for identifying the food additives and specifying the allowance criteria in food processes and they are indicated in the food labels. The list of antioxidants used as food additives is given in Table 1.9.

Table 1.9 List of antioxidants as food additives. (Turkish Food Codex Regulation, 2003)

E number	Definition	E number	Definition
E300	Ascorbic acid	E311	Octyl gallate
E301	Sodium ascorbate	E312	Dodecyl gallate
E302	Calcium ascorbate	E315	Erythorbic acid
E304	Fatty acid esters of ascorbic acid	E316	Sodium erythorbate
E306	Tocopherols	E319	Tertiary-butyl hydroquinone (TBHQ)
E307	Alpha-tocopherol	E320	Butylated hydroxyanisole (BHA)
E308	Gamma-tocopherol	E321	Butylated hydroxytoluene (BHT)
E309	Delta-tocopherol	E330	Citric Acid
E310	Propyl gallate	E586	4-Hexylresorcinol

There are many different E coded antioxidant food additives used in food industry. Some of them are more common, some of them are found naturally and some of them are obtained synthetically. Ascorbic acid which is also known as Vitamin C is available in various food sources in nature and it is a natural antioxidant. Vitamin C is used in the production of fruit juices. BHT and BHA are the two examples of the synthetic antioxidants used in foods.

They are commonly used in food products which are exposed to frying process, such as liquid fats, also in solid fats and in cereals, grains.

Although chocolate contains high amount of fat in it, no antioxidants are added to the recipe of the product during production process. This can be explained with the natural antioxidant content of the chocolate which comes with the cocoa content inside of it. Polyphenols in chocolate provide protective effect on the chocolate during its production steps and for its shelf life.

1.2.2 Classification of Antioxidants

Antioxidants are mainly composed of three different groups; enzymes, vitamins and phytochemicals. Plant antioxidants which can scavenge free radicals and prevent oxidation are effective examples

The enzymes are proteins and they come from our diet or synthesized in the human body. One of the most important antioxidant enzymes is superoxide dismutase (SOD) enzymes; however they require cofactors to provide antioxidant effect (Nutrex Hawaii, 2014).

The other group of antioxidants is vitamins. They are essential for human health and a diet rich in fruits and vegetables supply necessary vitamins. Most widespread vitamins found in foods are Vitamin A, Vitamin C, Vitamin E, folic acid, and beta-carotene (Nutrex Hawaii, 2014).

The last group is phytochemicals and they have three main sub-groups. These groups are carotenoids, polyphenols and ally sulfides (Nutrex Hawaii, 2014).

1.3 Polyphenols

Polyphenols are one of the largest groups of phytochemicals. They are classified as antioxidants and accepted as one of the most important type of

antioxidants exist in plants such as fruits and vegetables Black tea, green tea, red wine, cocoa, chocolate, blueberries, coffee, grapes, and grape seeds are widely consumed pholyphenol rich foods (Lee, Kim, & Lee, 2003).

Polyphenols are chemical compounds which contain at least more than one several hydroxyl phenolic groups. These phenolic groups contain at least two aromatic rings having six carbons. Compounds that have a six carbon ring are named as phenolic acids. They have the similar phenolic properties and characteristics (Weichselbaum and Buttris, 2010).

1.3.1 Classification of Polyphenols

According to their chemical structures, polyphenols are mainly grouped as phenolic acids, coumarins, flavonoids, isoflavonoids, stillbenes, lignans, and phenolic polymers. There are an enormous number of different plant phenolics and one of the most widespread type is flavonoids.

Flavonoids consist of the aromatic ring structure generally having the carbon rings as C6-C3-C6 (Bravo, 1998). They are widely distributed in foods and they have several important bioactivities. Figure 1.2 shows the flavonoids, their classification and some important examples (Craft et al., 2012).

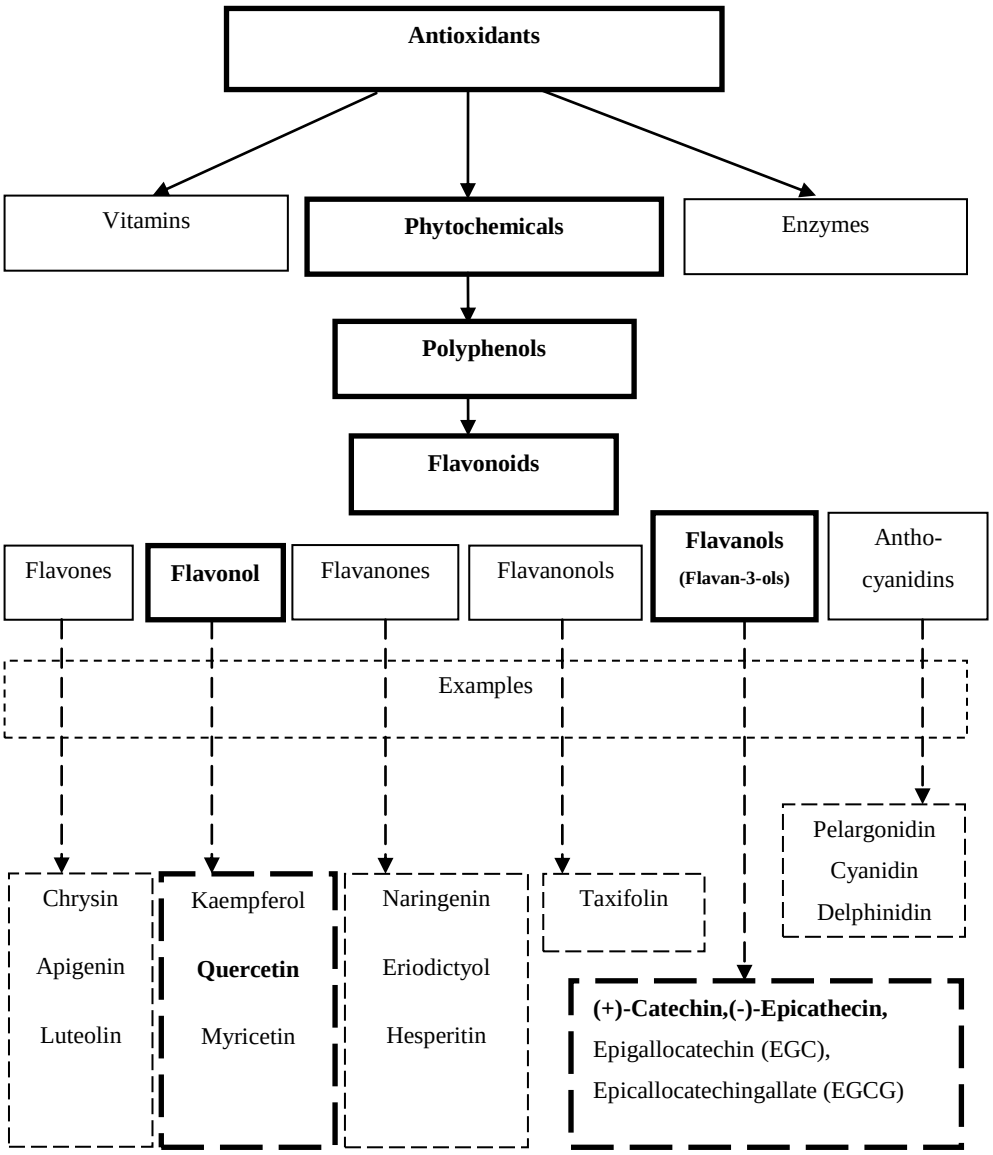


Figure 1.2 Classification of the antioxidants and polyphenols (Craft et. al., 2002)

In recent years, flavonoids have attracted great attention due to their beneficial effects on cardiovascular health. They have strong antioxidant potential therefore they can affectively scavenge free radicals produced in the membranes of cells lining the inner walls of blood vessels and they can prevent lipid peroxidation reactions that could cause plaque formation and

atherosclerosis. Figure 1.3 indicates the basic chemical structure of the flavonoids (Bravo, 1998).

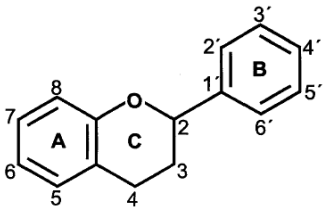
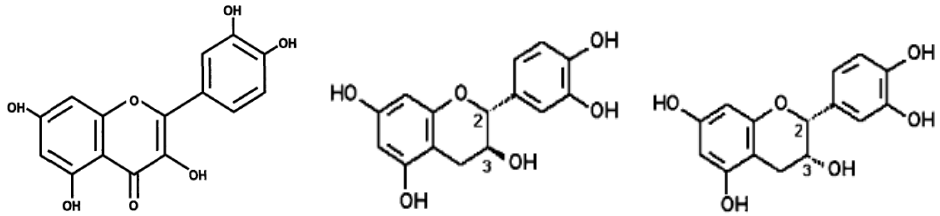


Figure 1.3 Basic chemical structure of the flavonoids (Bravo, 1998)

The classifications of the flavonoids are dependent on their chemical structure, and the number of aromatic rings (Weichselbaum, Buttriss, 2010). Figure 1.4 shows the chemical structure of the quercetin, catechin, and epicatechin (Dixon, Xie, & Sharma, 2005; Weichselbaum et al., 2010).



a b c

Figure 1.4 Basic structure of the (a) quercetin, (b) catechin, and (c) epicatechin (Dixon et. al., 2010)

1.3.2 Sources of Polyphenols

Polyphenols naturally exist in fruits and vegetables (Schenker, 2000). In plants, they are naturally synthesized by the help of light (Weichselbaumet al., 2010). Certain foods are very rich sources of polyphenols and cocoa produced from a cocoa tree fruit is one of the riches sources of polyphenols. Cocoa is the main ingredient of chocolate therefore chocolate is also a food source with high antioxidant content. Wine is another example which is a drink made up from grapes and it is also a very good source of polyphenols (Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012).

Table 1.10 Some examples are given for the sources of certain phenolic compounds (Weichselbaum et al., 2010).

Class of the Polyphenol	Name of the Polyphenol	Source of the Polyphenol
Flavonol	Quercetin	Broccoli, onions, cranberies, blueberries, cocoa powder
Flavanols (Flavan-3-ols)	(+)-catechin, (-)-epicatechin, gallocatechin	Red wine, chocolate, grapes, peaches, plums, rapberries, cherries, tea, apricots, cocoa
Hydroxybenzoic acid	Gallic acid	Very few edible plants; tea, raspberries, blackberries
Stillbenes	Resveratrol	Grapes, berries, peanuts, red wine

In addition to cocoa and wine, nuts, berries and green tea are also rich sources of different polyphenols. The availability of fruits and vegetables are dependent on the season and their shelf lives are short. However tea, cocoa and chocolate are easier to be found and consumed independent on the season. In addition, chocolate contains (-)-Epicatechin (EC) which is one of the most important flavanols with its high bioavailability. EC has been investigated with many researchers because of its health benefits on cardiovascular system. In recent years it has been derived from chocolate, especially dark chocolate, and cocoa. The amount of catechins in chocolate depends on the cocoa type, cocoa powder production steps and chocolate production steps. Even though the source of cocoa seems to be same, it is affected from variety a of conditions. Therefore, further research is needed to reveal the beneficial potential of bioactive compounds in food sources and the molecular mechanisms behind their bioactivity and the quantity of these phenolics in different food sources should be investigated.

1.3.3 Bioavailability of Polyphenols

The variety of foods contains the phenolic compounds by their nature. Table 1.10 indicates examples for the sources of certain phenolic compounds. There are two different terms related with the metabolism of antioxidants in the diet which are bioaccesibility and bioavailability. Bioaccesibility is the antioxidant content of the food substance which comes out after digestion. On the other hand bioavailability is the portion of these bioaccesible antioxidants which is absorbed by the body by the intestinal system (Weichselbaum et al., 2010). Therefore, how much we get from a food source is not the only indication of the bioaccesibility of those products. It is important to consider the daily intake of a nutrient together with its bioavailability. The daily intake of polyphenols is reported to be around 1 g / day. Bioavailability of polyphenols

is also related with the amount ingested by the food, its absorption from the intestine and its digestion (Haminiuk, Maciel, Plata Oviedo & Peralta, 2012).

Although there are a number of studies explaining the bioavailability of polyphenols, the mechanism is still required to be revealed (Haminiuk et al., 2012).

EC is one of the most important polyphenols found in chocolate. The bioavailability of EC is possible to be investigated by different methods and in vivo test. In a recent study, EC in plasma and urine was evaluated after the candidates consumed chocolate. The analysis was carried out by HPLC and LC/MS. After several hours, it was observed that the level of total EC increases and the maximum level in the plasma were reached after two hours of chocolate intake. It was recorded that 29,8±5, 3% of the total EC intake from chocolate consumption was excreted in the urinary system as EC metabolites, which indicates that EC is one of the most important highly bioavailable polyphenols presents in chocolate.

1.4 Polyphenols in Chocolate

Chocolate is one of the best sources of the antioxidants with its high cocoa content. Cocoa in chocolate provides a considerable amount of flavonoids. It has more antioxidants than tea and wine (Ali to et. al, 2004).

Flavonoids are plant pigments and 6500 different flavonoid structures are known. They have different subclasses such as flavones, flavanones, flavonols, flavanols, anthocyanidins, and isoflavones (Weichselbaumet al., 2010) and flavanols attracted important attention due to their bioactivities. Flavanols found in chocolate has mainly three different groups C, EC and procyanidins. The most significant group is EC and C in terms of bioavailability.

Figure 1.5 shows the structure of major flavanols which are found in chocolate due to its cocoa content (Afoakwa, 2010)

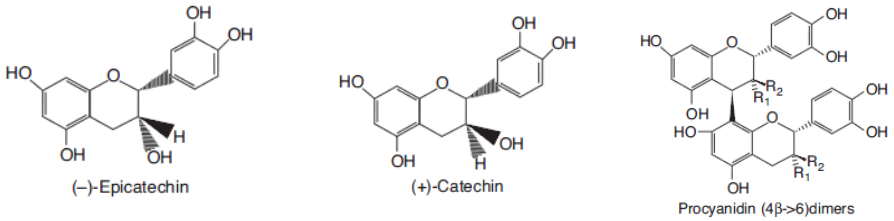


Figure 1.5 Major flavanols which are found in chocolate due to its cocoa content

Polyphenol content of chocolate is significantly related with to its cocoa content. Darker chocolates are supposed to have more antioxidant capacity than the less cocoa containing ones such as milk chocolates. It is also investigated that cocoa content in the chocolate is not the only factor which affects the antioxidant capacity. Other ingredients such as milk in chocolate also have inverse impact on the bioavailability of polyphenols in chocolate (Ali, Ismail, & Kersten, 2014).

There are different methods which provide measurement of the available antioxidant content. According to Waterhouse et al. (1996) milk chocolate has less antioxidant content than the dark chocolate. He made several analysis about the antioxidant capacities of milk chocolate and red wine. According to him, even though the standard milk chocolate is worse in antioxidant content than the standard dark chocolate, 45 g standard milk chocolate and 150 ml red wine have approximately the same amount of polyphenols (Schenker, 2000), which can be regarded as a very significant result.

Cocoa has polyphenol content around 6-7% of dry cocoa beans. When a regular American diet is analyzed, chocolate are the most important antioxidant sources after fruits and vegetables (Ali et al., 2014).

Polyphenols in chocolate are able to reduce the free radicals, they can help to stabilize membranes and they have a property which is known as free radical scavenging activity (Afoakwa, 2010).

In addition to polyphenols, fat containing cocoa solid material which is known as cocoa butter is also regarded as healthy compared to other fats. Fats found in cocoa are mainly stearic triglycerides (C18:0). They have more tendencies to be excreted by faeces. Strearic triglycerides' bioavailability is less than the other fats and has lower effect on cholesterol increase (Wollgast & Anklam, 2000b as cited in Afoakwa, 2010).

1.5 Potential Benefits of Chocolate Polyphenols for Human Health

Statistical data shows that death due to cardiovascular diseases increased in recent years. According to the 2012 reports of WHO, while 17 million people died because of the cardiovascular diseases in 2008, it is predicted that this number will increase to 25 million in 2030, and while 7.6 million people died due to cancer, this number will increase to 13 million. The body is composed about what we eat, and eating habits are one of the most important reasons of development of these diseases. It is significant to consume foods which are helpful to improve and maintain our health.

One of the reasons of cardiovascular health problems is the adverse effects of free radicals in our body. Recent researches are focused on decreasing the free radicals with antioxidant dietary food sources. Cocoa, tea, green tea, coffee,

chocolate, grape, grape seeds, and berries have impressively high amount of antioxidants helping to inhibit those free radicals. In addition, polyphenol-rich food consumption habits decrease the risk of death related with chronic hearth and cardiovascular diseases (Jargic, 2002). It is reported that a diet including necessary dietary components have a critical prevention or management effect on important diseases such as cardiovascular diseases, neurological diseases, high cholesterol levels, high blood pressure and obesity (Zumbe, 1998).

1.5.1 Chocolate and Cardiovascular Health

According to World Health Organization (WHO), cardiovascular diseases are the first reason of death worldwide and the number of cardiovascular disease increased dramatically in recent years. It has been well established that life style and nutrition has an important impact on human health and wellbeing. Epidemiological and food intervention studies have revealed that consumption of polyphenol rich foods such as fresh vegetables and fruits affect human health positively. The most widespread polyphenol sources foods are pomegranate, grape, cranberry, tea, and cocoa. Especially cocoa contains high amount of flavonoids that are bioactive compounds among polyphenol group.

Polyphenols mostly coming from cocoa in chocolate has various health related functions. The benefits related with heart and cardiovascular health, and blood pressure is related with the given mechanisms below;

- Free radical scavenging activity
- Inhibition of lipid peroxidation
- Providing resistance to oxidative stress

Related health benefits of chocolate polyphenols are listed in Figure 1.6.

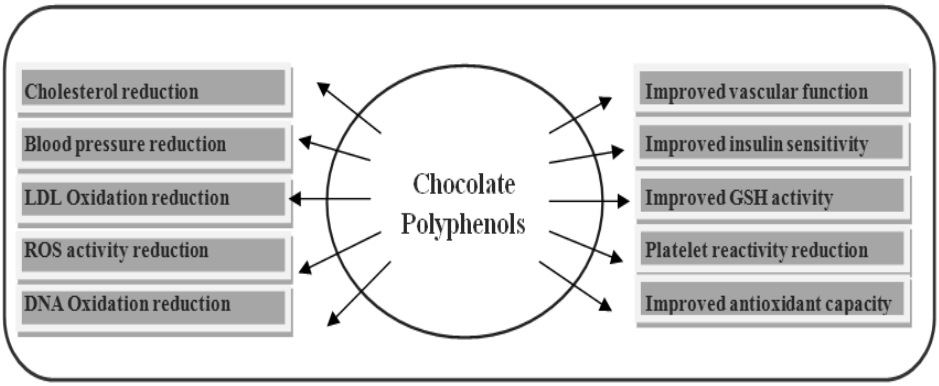


Figure 1.6 Health Benefits of Chocolate Polyphenols

1.5.2 Chocolate and Cardiovascular Health (Blood Pressure)

Recent studies indicate that there is a positive correlation between consumption of antioxidant rich foods and the cardiovascular health (Latif, 2013). According to Zomer (2007), daily consumption of dark chocolate provides prevention of cardiovascular diseases.

The health benefits chocolate mainly comes from cocoa antioxidants and their blood pressure lowering effect. The mechanism behind these benefits is associated with the high antioxidant capacity of cocoa. Increasing oxidative stress caused by free radicals in blood causes formation of plaque inside the arteries which may results in atherosclerosis. Oxidized cholesterol and lipid particles deposited inside the blood vessels are the main causes of atherosclerosis. As a free radical scavenger, chocolate plays an important role in scavenging of free radicals and inhibition of lipid peroxidation in blood vessels (Latif, 2013), conseuqentially, it can prevent the formation of plaque and vascular damage. Experimental, observational, and clinical research revealed that there is a correlation between chocolate consumption and lower risk of cardiovascular diseases. According to Latif, people who regularly

consume dark chocolate in their daily diets, have a lower blood pressure (Latif, 2013). Low blood pressure is very important to have lower risk of heart diseases and a healthy cardiovascular system. In a study carried out by Andujar et al. (2012) with 136 candidates, cocoa and chocolate is proved to have a beneficial and positive effect on cardiovascular system. There are several different mechanisms that provided this health effect (Andujar, Recio, Giner & Rios, 2012). These mechanisms are decreasing LDL level, increasing HDL level, lowering the blood pressure, decreasing the risk of platelet formation, decreasing the inflammation in the body. All of them provide healthier cardiovascular system (Andujar et al., 2012).

1.5.3 Chocolate and Brain Health

Chocolate has highly positive impact on brain health and this is mainly thought to be related with the blood flow improvement and its correct flow through the brain. This mechanism is thought to provide better memorial and thinking ability. These benefits were studied in Harvard Medical School and adults were provided to drink two cups of cocoa which contains chocolate polyphenols. In another similar study carried out in 2012 in Italy with older adults, the idea that beneficial actions of polyphenols on brain health may be related with the hypertension was supported. These results indicated that blood pressure lowering effects of cocoa polyphenols helped improvement of thinking ability and memory. However none of these studies exactly proved that there is clear and strict relation for protection of brain by cocoa and chocolate. They could only provide a positive relationship of chocolate polyphenols and brain health (Harward's Woman Health, 2014). Further research is needed to clarify the molecular mechanisms of beneficial effects of polyphenols on human health.

1.5.4 Phenolics from Chocolate in Cancer Prevention

Antioxidants in cocoa and chocolate have many beneficial effects on human health. They protect from the development of diseases by reducing oxidative stress, which is the main contributor to the diseases. Free radical scavenging activity of polyphenols play a critical role also in cancer prevention and therefore chocolate polyphenols and their antioxidant activities attracted important attention in cancer prevention and treatment strategies (Andujar et al., 2012). According to Zumbe (1998) in vitro studies of mammalian cells are supporting the beneficial effects of polyphenols. In that study, catechin, epicatechin, quercetin, gallocatechin, and epigallocatechin were reported to have a potential to prevent organisms against cancer (Zumbe, 1998). Polyphenols protect the cells against oxidative stress and this is one of the most important mechanisms for cancer development and progress.

Andujar et al., (2012) have applied cocoa extracts on cells and showed that cocoa antioxidants decreased cellular oxidative stress effectively. Another protection mechanism of cocoa polyphenols is related with their effects on DNA. There is well established correlation between DNA damage and mutagenesis and carcinogenesis. Chocolate polyphenols play role in DNA protection mainly related with ROS scavenging activity and inhibition of mitomycin C initiated DNA inducer activity (Andujar et al., 2012). Even though these studies show that there is inhibitory effect of chocolate polyphenols on cancerous cells, the exact mechanism behind this anticancer effect is still not clear enough (Latif, 2013).

1.6 Aim of the Study

The aim of this study is investigating the antioxidant content and activity of dark and milk chocolates in Turkish market and comparing them with other chocolate researches in the literature. It is important to investigate the amount of available antioxidant in chocolates in order to understand how much consumption of a chocolate provides how many amount of antioxidants as a daily intake.

In order to evaluate the antioxidant activity and capacity of the samples, 5 methods are applied. They are DPPH and ABTS for antioxidant activity determination, Total Phenol Content, Total Flavonoid Content, and HPLC. According to the results of these methods, the comparison of the samples and their comparison with literature results belonging to other countries' investigations is the main objective.

CHAPTER 2

MATERIALS AND METHODS

In this study, chocolate samples obtained from Turkish market were analyzed for their free radical scavenging activities, total phenolic and flavonoid contents and their EC and C contents were determined by using RP-HPLC analysis.

2.1 Materials

2.1.1 Chemicals

Chemicals used for this study were obtained from Merck and Sigma.

Chemicals used as the flavonoid standards were quercetin, catechin, trolox, and epicatechin were procured from Sigma and Gallic acid was bought from Merck.

The other chemicals, DPPH● and ABTS●, which were used as oxidants or radical species, were obtained from Sigma.

Potassium persulfate, aluminum chloride, sodium nitrite, methanol were the other chemicals which were used as process aid material during the methods and they were provided from Merck. Ethanol was obtained from Sigma. Chromotographic acetonitrile, TFA, THF, and water were obtained from Merck in HPLC grade.

2.1.2 Equipments

In this study different methods were used to investigate the antioxidant activity and to understand the phenolic and flavonoid content of the samples. In order to prepare the solutions the glass and plastic equipments were used. 0.45 μm non-pyrogenic filters were used to clarify the extracts used in chromatographic analysis. Magnetic stirrer and vortex were used in order to provide perfect mixing at different stages.

Spectrophotometer was used for making antioxidant activity measurements of extracts. Micropipettes and plastic pipettes were used for dilution of the sample solutions. For the chromatographic analysis RP-HPLC was used. The HPLC equipment consisted of a Shimadzu LC-20AD system including DGU-20A5 prominence degasser, SIL-20AHT prominence auto sampler, SPD-M20A prominence UV-Vis photodiode array detector, CTO-20A prominence column oven. Agilent Zorbax SB-C18 (250 mmx 4,6 mm, 5 μm) column was used for chromatographic separation. Diode Array Detector (280nm) was used as detector. 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 was used and it was consisted of two solvent solutions A and B. A was water: tetrahydrofuran: trifluoroacetic acid (98:2:0.1, v/v/v) which was a polar solvent, B was acetonitrile**:** trifluoroacetic acid 0.1 % TFA solution in acetonitrile. Data was processed on an Intel Pentium IV PC computer by using LC Solution Programme.

2.2 Sample Selection

There are many different chocolate brands in Turkey markets, and for this study different most popular chocolate producers were chosen. The reason

behind choosing samples from different brands was that process type, process conditions, ingredient properties, cocoa and chocolate liquor properties differs from brand to brand. In addition to this, chocolate types are related with their cocoa solid content and additional milk content.

In this study milk and dark chocolates were chosen for antioxidant analysis and white chocolate was not used because in the literature it was reported that the chocolate polyphenols come from the non-fat cocoa solid content in chocolate which was not used in the production of white chocolate. It was expected that chocolates with high cocoa content should also high antioxidant activity related with their polyphenol contents.

Commercially available different milk chocolate samples (n=3) and different dark chocolate samples (n=3) were selected, with a total sample number of 6. They were chosen from the best seller brands from the markets and it was considered that these chocolates were produced in Turkey.

Samples were grouped as shown in Table 2.1. Chocolates were grinded and stored at -18C till defatting step

Table 2.1 Chocolate samples and their classification.

Type	Sample Naming	Cocoa Solid (%)	Fat (%)
Milk Chocolates (MC)	MC1	29-33	32-36
	MC2	27-31	28-32
	MC3	28-32	29-33
Dark Chocolates (DC)	DC1	68-72	32-36
	DC2	49-53	30-34
	DC3	78-82	38-42

2.3 Sample Preparation

2.3.1 Grinding

Samples for this study were block chocolates and in order to make analysis it was necessary to increase the surface area. For this purpose chocolates were grinded. These sample were then stored at -18ºC until defatting. The picture of the samples after grinding is shown in Figure 2.1.

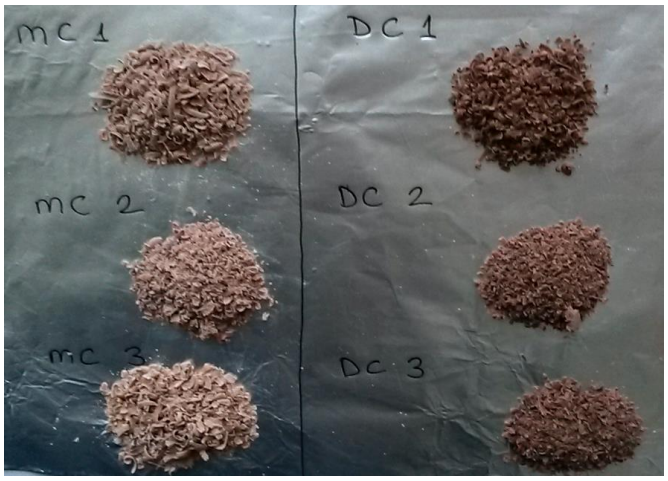


Figure 2.1 Milk chocolates (MC1-MC2-MC3), Dark chocolates (DC1-DC2-DC3).

2.3.2 Defatting

Chocolate has very high fat content which comes with cocoa butter. In general white chocolate contains cocoa butter and milk and for dark chocolates there is at least around 30% fat. The polyphenol content in the chocolate is related with the non-fat cocoa solid content of it (Gu, House, Wu, Ou, & Prior, 2006). Thus,

the fat content in the chocolate should not be taken into account. In brief, the purpose of defatting process was eliminating any impurities or deviations in the measurements caused by different molecules read on spectrophotometer during the antioxidant activity analysis. Defatting was done with hexane (Gu et al., 2006). Indeed this defatting process can be considered as fat extraction from the chocolate samples. While deciding the defatting procedure, literature research was guided. Chocolate samples which were prepared as grinded form and stored at -18°C were used for defatting. The ratio between grinded chocolate and hexane was chocolate: hexane; 1:10, v/v. Hexane as 200 ml was poured into an Erlenmeyer flask and 20 g grinded chocolate was added on it. They were mixed well by vortex for 30 sec and then put into the shaker at 110 rpm speed and 25°C and they stayed in the shaker for one hour. After an hour two different phases occurred as fatty side and chocolate solid side. In order to make most efficient defatting, the fatty side was pipetted out and filtered through a filter paper. Then the second hexane addition was done on the solid mass side which was remained in the Erlenmeyer flask as 200 ml again. Then it was vorterxed 10 sec again. The sample was put into the shaker for an hour at 25°C and 110 rpm. After this second application, the mixture had two different phases, fatty liquid part on the top and more solidified part at the bottom. The fatty solution side which was floating on the top was again pipetted out as the same way with the first defatting. This pipetted solution was filtered and the remaining non fat chocolate solid on the filter paper was collected after totally dried. The more solidified part which had the most of the non fat chocolate solid of the mixture was staying at the bottom side of the Erlenmeyer. In order to remove the fat in this part it was left for drying. During drying the hexane was removed from the mixture and when it was totally dry, it was scrapped away from the Erlenmeyer flask. This removed part of the chocolate is the defatted chocolate Figure 2.2 presents the samples of grinded chocolate in the hexane and Figure 2.3 shows the grinded and defatted chocolate samples.

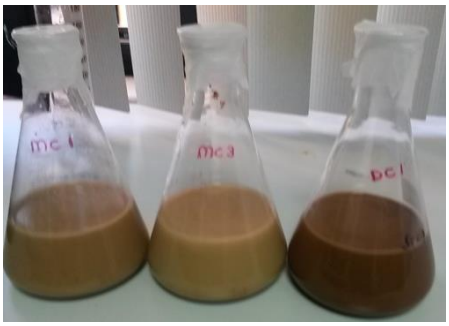


Figure 2.2 Grinded chocolate in the hexane; chocolate: hexane solution (1:5) before completing defatting

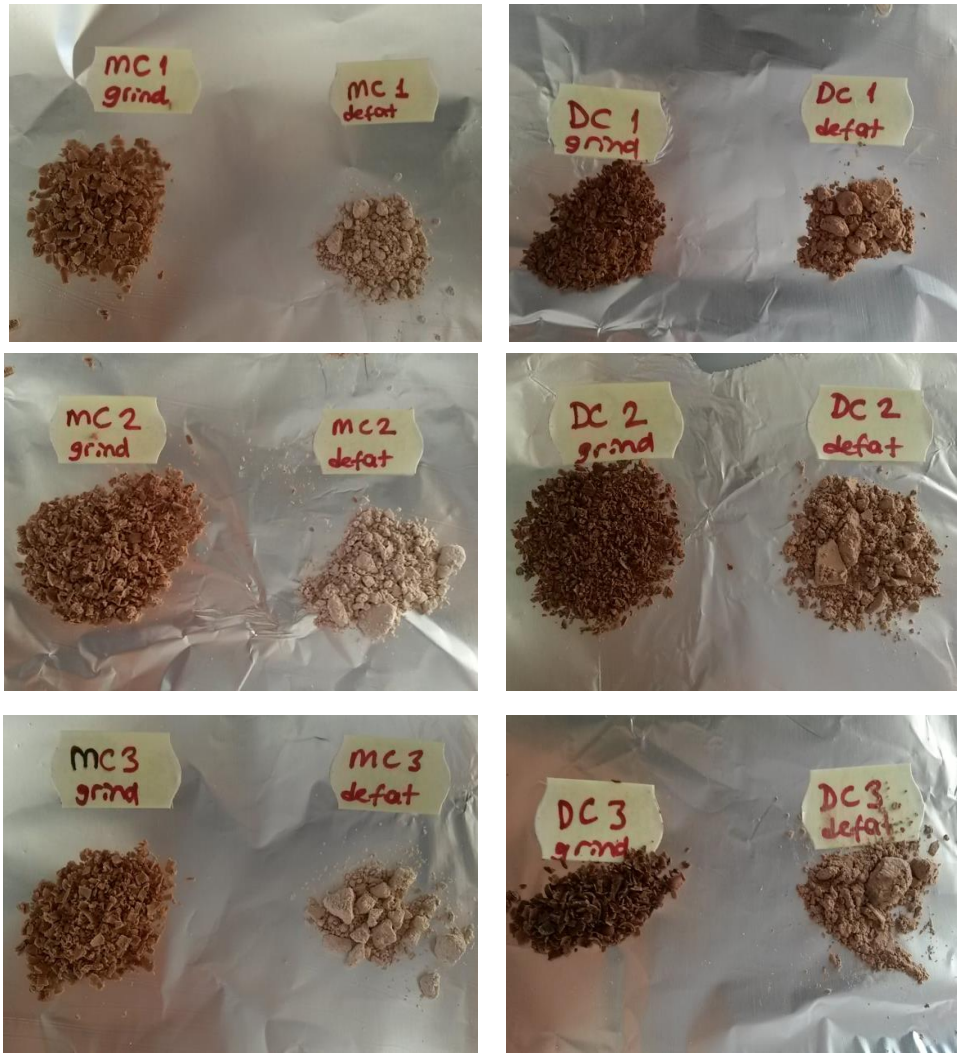


Figure 2.3 Grinded and defatted chocolate samples.

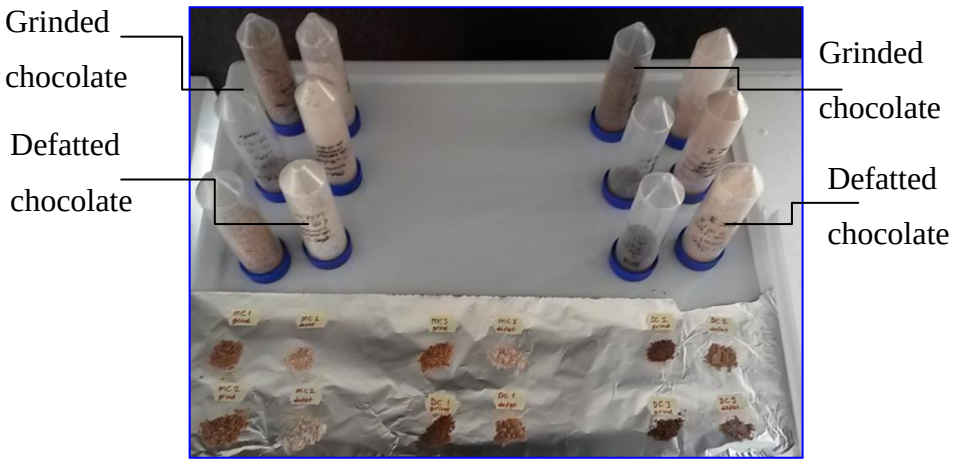


Figure 2.4 Grinded and defatted chocolate samples.

2.3.3 Extraction

In order to make more efficient analysis related with the antioxidant activity of the defatted chocolate, it was required to prepare them as solutions with proper solvents. At this stage, in order to provide the polyphenols in the chocolate samples extraction process is used. The methods for antioxidant activity and the polyphenols content of the chocolate are valid for the liquid solutions of the non fat chocolate samples. For the most effective extraction through the solvent, suitable solvent selection is significant. For extract of the defatted chocolate samples, different methods were possible. It depends on the next analysis such as antioxidant activity analysis or sugar content or etc. For this part, literature research was done for samples which were subjected to the phenolic content analysis or antioxidant activity analysis. Sample type was another indicator which defines extraction method. Cocoa and chocolate extraction and analysis for antioxidant activity is almost the same in most of the cases.

The main research which was also used as the reference for most of the further research was belonging to the Adamson method (1999). The last modification

on this method was done in 2007 (Cooper, Gimenez, Alvarez, Nagy, Donovan, &Williamson, 2007) and this was applied for the analysis of the cocoa polyphenols.

Extraction was applied on the defatted chocolate samples and acetone: water: acetic acid (70:28:2) was used as solvent for extraction process.

4 g of each of sample were weighed and labelled (MC1, MC2, MC3, DC1, DC2, and DC3). Then they were put into the plastic flasks and solvent was added on them in 1:5 sample to solvent ratio. 20 ml of solvent was added on 4 g defatted chocolate samples. Then, this mixture was subjected to the vortex for 30 sec. and shaker was used for perfect mixing and providing extraction. Samples were left in the shaker at 110 rpm at 30°C. They were left for overnight and then they were separated as two phases which is shown on the Figure 2.4.

Figure 2.5 Extracts after overnight incubation in the shaker

The samples' liquid parts were filtered with a filter paper. The remaining solid part of the mixture was extracted for the second time during overnight at the same conditions with the addition of the same amount of the solvent on and this provided double extraction. After this step the samples were again filtered. The solution after the filter paper was the first extracted part which contains the

chocolate polyphenols and they were homogenous in the solution after this extraction step. The remaining part which contains the insoluble chocolate solid content was weighed and calculated and extraction yields were determined.

This extraction process is explained with a flowchart given in the Appendix A-1.

2.4 Methods of Analysis

Recent studies are concentrated on the chocolate and its health benefits in literature. However there is not any study conducted on the chocolate which are sold in Turkey. In this study the main objective is analyzing the antioxidant capacity and activity of the chocolate which are consumed in Turkey. Chocolate health benefits mainly come from its cocoa content and thus, the quality of a chocolate and beneficial part depends not only on the process conditions but also on the cocoa and chocolate liquor in it. Because of these reasons, the researches done on the different chocolates which are consumed in different countries does not reflect the results for chocolates consumed in Turkey.

In this study, chocolates which are extracted and prepared as serially diluted are examined with different methods.

2.4.1 Solvent Selection

Different solvents are used for the most efficient analysis of the antioxidant content and antioxidant activity of different samples for cocoa and chocolate there are various solvents but one of them is more widespread for recent researches.

According to Adamson extraction method (Adamson, 1999), which is known as the main skeleton of the extraction method for the antioxidant analysis, was done by the solvent having acetone, water and acetic acid with the ratios of 70, 29.5 and 0.5 respectively. On the other hand the other research (Lee et al., 2007) was done by water only. The other method in another research analyzing the antioxidant property of polyphenols rich products was done by only acetone and water with 70 and 30 ratios respectively (Schinella, Mosca, Jovellanos, Pasamar, Muguerza, Ramon, & Rios, 2010). The other method is that the usage of the acetone, water and acetic acid with the ratios of 70, 28, and 2 respectively (Cooper et al., 2007). This method was done with some adjustments on the Adamson method and it can be considered as the most recent version of the Adamson method.

In this study, solvent optimization was applied and there were three different solvents used at the beginning. The analysis had been applied on one sample which was DC3 with three different solvents. These solvents were water, acetone-water, and acetone-water-acetic acid. Table 2.2 explains the usage of the acetone, water and acetic acid with different ratios for different solvents

Table 2.2 Solvents used for the extraction process and their ratios

Solvent	Acetone	Water	Acetic acid
Solvent 1	70	28	2
Solvent 2	70	30	0
Solvent 3	0	100	0

These three solvents were applied for extraction and then the methods for analysis were done on DC3 sample for optimization. DPPH$^{\bullet}$, ABTS$^{\bullet}$, total phenolic content determination and total flavonoid content determination methods were applied on these three different solvent dissolved samples which did not have any difference from the beginning until the mixing with a solvent. The results and the literature research results has been used together in order to get the most optimized results from the analysis.

2.4.2 Antioxidant Capacity Determination

In order to determine antioxidant activity as a free radical scavenger there are different methods and DPPH radical scavenging activity and ABTS radical scavenging activity are the common ones. DPPH● is not an enough indicator for antioxidant analysis alone. Thus, ABTS● and DPPH● were generally made together for a sample to determine the free radical scavenging activity.

2.4.2.1 DPPH● Radical Scavenging Activity

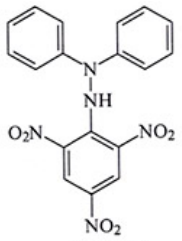
DPPH● is one of the free radicals which has a chemical name 1, 1-diphenyl-2-picrylhydrazyl. In this method, the antioxidant activity is measured by the radical scavenging activity of the chocolate extracts in DPPH● solution (Wiliams, Cuvelier, &Berset, 1994).

DPPH● is in its powdered form and for this method, it was used as a solution because of that it should be calculated from its absorbance value on the spectrophotometer. This method was also used in Cooper's study with some adjustments (Cooper et al., 2007). Figure 2.6 presents the chemical structure of DPPH Free Radical and its solution in ethanol.

Figure 2.6 Chemical structure of DPPH Free Radical and its solution in ethanol

The applied method was adapted from the Blois method which was published in 1958 (Blois, 1958). The first step of this method was the preparation of the DPPH● solution. The DPPH● powder was dissolved in the ethanol in order to produce free radical DPPH●. The absorbance of this solution should be 1.4 at 517 nm wavelength. This solution was the stock solution of the DPPH • and it was purple colored. In general the DPPH● stock solution is very sensitive to light, thus; it should be kept in dark place without any sunlight and during the measurements on spectrophotometer it should be also as possible as dark in the laboratory. Then, 0.1 ml of the extract was added on the 1.4 ml of DPPH● stock solution in the plastic tubes. Tubes were put on the vortex and then they were kept in the dark at 23°C for 20 min. This procedure was applied for all the solutions as 4 replicates for all the dilution rates. Four replicates refer to 2 different measurements applied as doubles of both of the time. After 20 min, samples were measured by spectrophotometer. Absorbance values were recorded which were measured at 517 nm wavelength. Blank tube was also applied and these spectrophotometric results were used for the IC50 calculations. IC50 indicates the required antioxidant activity in order to reduce the 50% DPPH● radical.

The extracts are shown on Figure 2.7-8 after the reduction of DPPH●.



DPPH-H
(colorless)

Figure 2.7 Chemical structure of the reduced DPPH● and its appearance as a solution after addition of the chocolate samples

Figure 2.8 Appearance of reduced DPPH● and as a solution after addition of the chocolate samples

DPPH free radical scavenging activity was measured according to the spectrophotometric results. The radical scavenging activity (RSA %) was calculated by ABS at 517 nm and the final concentrations of the extracts. The reaction principle providing this scavenging activity is shown on the Figure 2.9 (Lee et al., 2007).

According to this reaction when the DPPH● radical reduced, the color of the solution becomes lightened and absorbance value becomes lower. The RSA%

and the final concentration curves were drawn according to this decrease in absorbance value. The RSA value and the concentration gave the IC50 value of the extract which is known as half maximum inhibitory concentration of the sample and reduces the 50% of the DPPH● free radical.

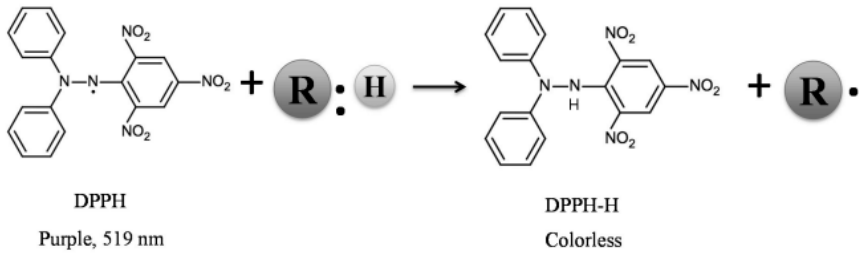


Figure 2.9 DPPH●(2,2-diphenyl-1-picrylhydrazyl) and its reaction with antioxidant. R: H represents antioxidant as a radical scavenger and R represents antioxidant radical.

2.4.2.2 ABTS● Radical Scavenging Activity

In ABTS● (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) method antioxidant activity is measured by reducing the ABTS radical. Trolox in this method is used as the standard which gives the standard curve for the calculations. The main difference between these two antioxidant activity measurement methods is that, while in ABTS● method, free radical is produced by the analyst by making ABTS● stock solution, for DPPH● method, DPPH● is a free radical already and it is only dissolved in ethanol in order make it ready for the analysis. ABTS• chemical structure is presented in Figure 2.10 (Dixon, Xie, & Sharma, 2004).

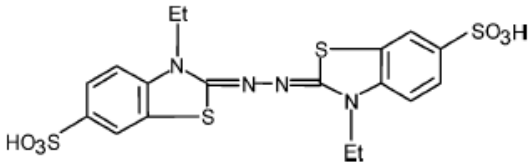


Figure 2.10 ABTS• chemical structure 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)

ABTS stock solution was prepared by the addition of 2.45 mM $K_2S_2O_8$ and 7 mM ABTS● powder with 1:1 ratio. This solution was kept in dark for 16 hours. It was the stock solution for further analysis in this method. ABTS● free radical stock solution preparation steps and radical scavenging activity measurement method steps are given in the appendix part (Appendix A-2). On the other hand Trolox which is a water soluble analog of Vitamin E, was used as the standard for this method and the chemical structure of Trolox is presented in Figure 2.11 (López-Alarcón, Speisky, & Lissi, 2007).

For this analysis, trolox solution should be in the interval as 25-600 µM for standard curve Figure 2.12 presents the sample of Trolox serial dilution and their reaction with ABTS● solution.

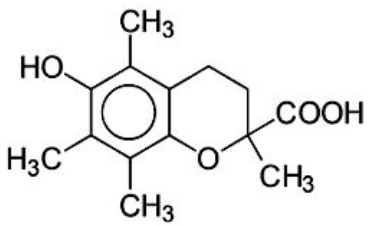


Figure 2.11 Chemical structure of Trolox

Results of the reaction of Trolox and ABTS● radical were measured by spectrophotometric analysis. The color of ABTS● stock solution is dark blue and while reacting with an antioxidant color changes through lighter blue to white. If the concentration of the antioxidant is higher, the color is lighter and the absorbance is lower in spectrophotometer.

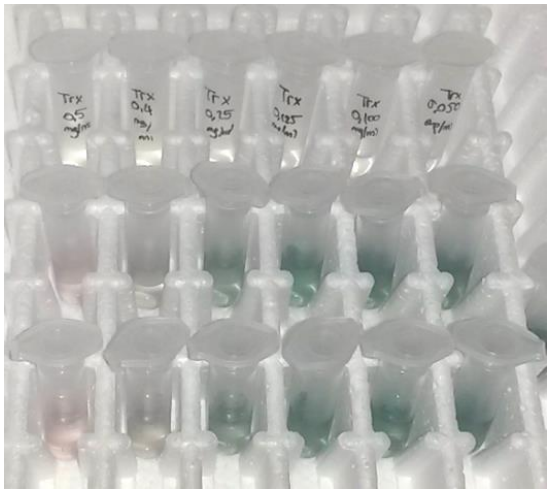


Figure 2.12 Trolox serial dilution and their reaction with ABTS● solution

Before starting the analysis the ABTS● stock solution should be diluted with methanol and this diluted solution should have 0.70 ±0.01 absorbance value at 734 nM. This solution is highly unstable and easily effected from the light. Thus, it always should be kept in dark (Arnao, Cano, & Acosta, 2000) and this solution should be prepared fresh for each time when the analysis starts.

For antioxidant activity determination, 10 µl of extract or Trolox solution was piped in the tube and 1000 µl of the ABTS● solution was piped on it. This mixture was put on the vortex and then incubated in dark for 6 min at 30°C. Then the mixture was read by spectrophotometer at 734 nM and the absorbance values were recorded. This measurement was doubled for two times. The

recorded absorbance values of trolox solution gave the standard curve and the absorbance values which were obtained from the extracts were put on the standard curve. After the calculations, trolox equivalence values were calculated as mg TEAC per mg extract. This result explains the antioxidant activity of the chocolate samples in terms of trolox equivalence. The free radical scavenging activity measurement method steps are given in the appendix part. (Appendix A-3)

When the reactions completed the absorbance values were used for standard curve and calculation of TEAC values. The equation for TE values of the samples was obtained from the trolox.

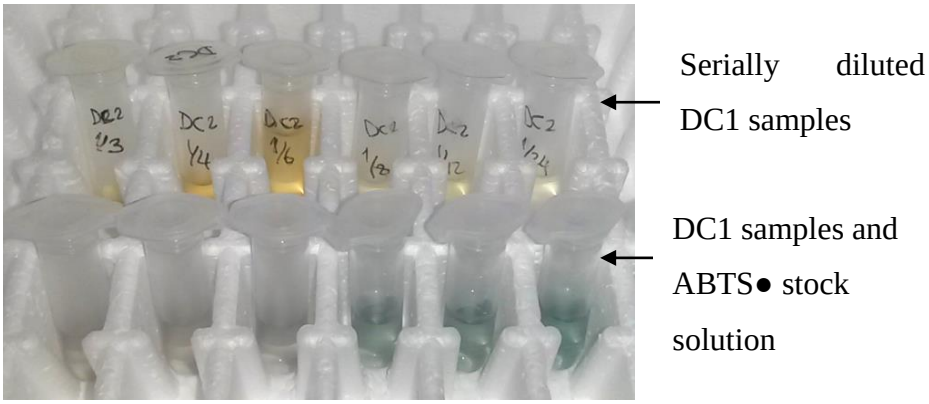


Figure 2.13 DC2 samples with different concentrations and their reaction with ABTS● solution

Figure 2.13 represents the serially diluted DC1 sample and their reaction with ABTS● stock solution. Because of the ABTS● free radical is induced more with more concentrated sample, the color becomes lighter. This color change gives absorbance values for TE calculation.

2.4.3 Total Phenol Content Assay

Total phenolic content of the chocolates in this study were analyzed by the Folin-Ciocalteu Method. Total phenol content was investigated by Singleton and Rossi in 1965 (Singleton at al., 1965) and many of the further researches have been done by using this method with some adjustments. In this method Gallic acid which is one of the phenolic acids was used as the standard. For the measurements the method was applied first on the Gallic acid solution and then this measurement results gave the standard curve for the calculations. Same method was also applied on the samples and then the calculations for samples gave the results which were explained as mg Gallic acid equivalence per mg extract according to the standard curve.

Gallic acid solution was prepared as the first stage of the method. Powder Gallic acid was dissolved in the solvent which was the same with the extraction solvent (acetone/water/acetic acid). 20 mg Gallic acid was added on the 50 ml solvent. The concentration of the solution became 0.4 mg GA / ml solvent. This solution was diluted and arranged with different concentrations like 0.4, 0.3, 0.2, 0.1, 0.05 mg/ml. These concentrations were the diluted stock solutions and were used for the standard curve. The following figures, which are Figure 2.14 and Figure 2.15, present the Gallic acid Solution in acetone/water/acetic acid solvent with different concentrations and the chemical structure of Gallic acid, respectively.

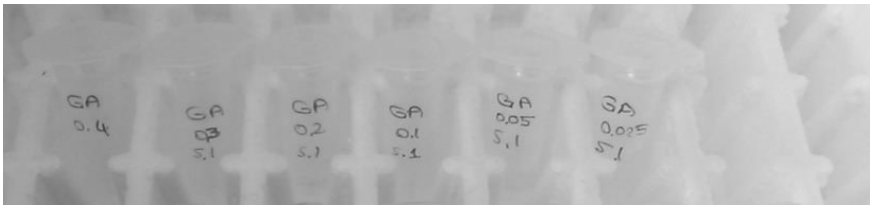


Figure 2.14 Gallic acid Solution in acetone/water/acetic acid solvent with different concentrations

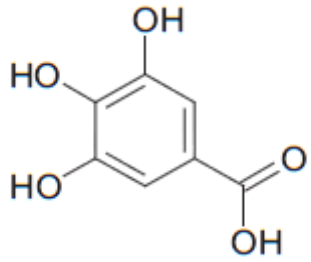


Figure 2.15 The chemical structure of Gallic acid

As the process aid of the method 2% Na_2CO_3 solution was prepared by adding 2 g Na_2CO_3 on the 100 ml distilled water. In addition 50% Folin reagent which is a radical was also prepared and used for the analysis.

Serial diluted Gallic acid solutions and extracts were ready and 0.15 ml of each was piped out into the tubes. Then 3 ml of the 2% Na_2CO_3 solution was added on. They were put on the vortex for 15 sec. and then kept at dark for 3 min. Then 0.15 ml of 50% Folin Reagent was added on this solution and again the tube was put on the vortex. After vortex, the solutions in tubes were kept in dark area for 30 min. Then they were measured by spectrophotometer at 750 nm relatively to the blank solution which was prepared by only solvent without any extract or Gallic acid.

The solution which was more concentrated in terms of the extract reduces more Folin reagent. The color of the solution becomes darker in the more concentrated samples because of the reduced Folin reagent.

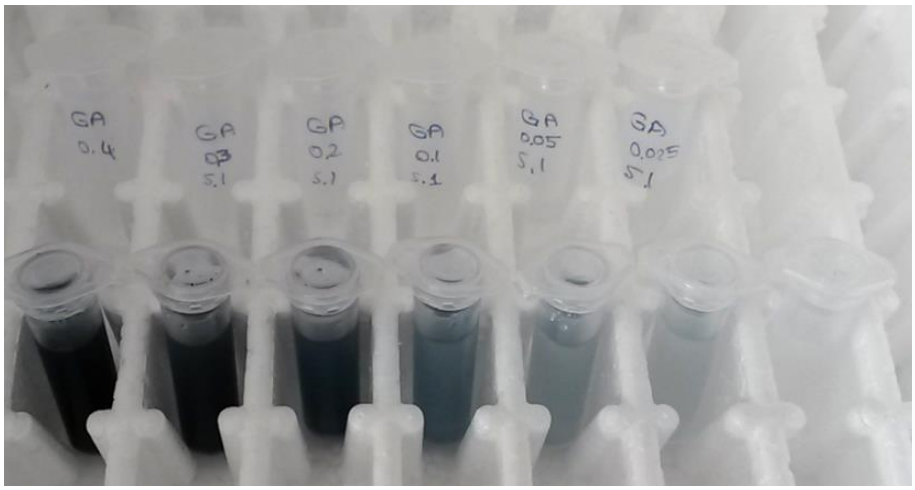


Figure 2.16 Serially diluted Gallic acid reacts with different amount of Folin reagent

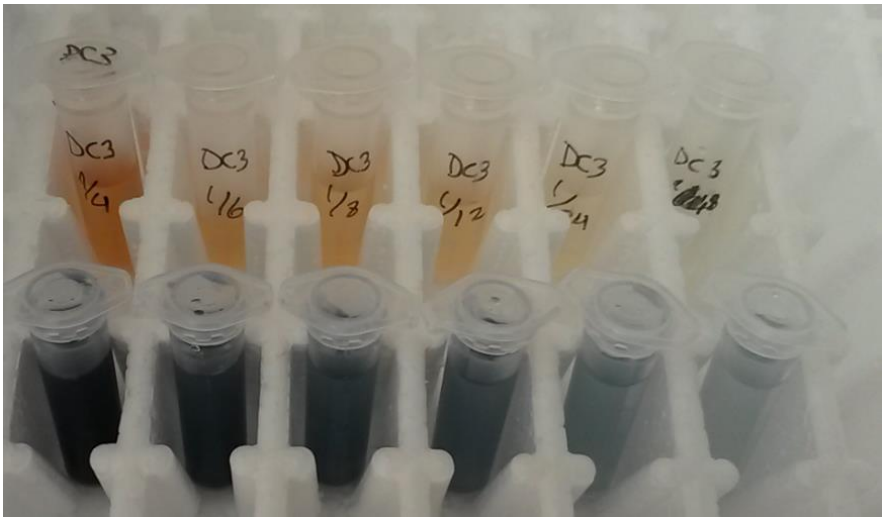


Figure 2.17 Folin method applied samples with different concentrations. More concentrated. High concentrated samples react with more Folin compounds

According to Figure 2.16 and 2.17 Folin reagent is the radical and Gallic acid or sample reacts with Folin reagent in order to induce. The Gallic acid was prepared by serial dilution in order to draw the standard curve. The samples were also prepared by serial dilution and the reaction is spectrophotometrically measured with the color difference.

The main mechanism in this method is related with the reduction of the Folin reagent. The phenolic substances react with the Folin reagent and this antioxidation activity reaction only can occur under the basic conditions. Na_2CO_3 solution provides this basic condition in the mixture. Under basic conditions, phenolic compounds dissociate and this dissociated form reduces Folin-Ciocalteu reagent. This reduction mechanism results with blue colored solution. This blue color provides measurement by spectrophotometer and the measurement gives reduction rate and total phenolic material which provides the reduction. The steps for standard solution preparation and reaction have been shown in appendix part (Appendix 1-4).

This absorbance value was used for both standard curve preparation and for calculations for total phenolic content in terms of Gallic Acid equivalence according to this standard curve.

2.4.4 Total Flavonoid Content Assay

Flavonoids are a subclass of the polyphenols and there are different flavonoids which also includes (+)-catechin, and (-)-epicatechin. Quercetin is the other flavonoid which is found in flavonols subclass. In this method, the total flavonoid content of the chocolate samples is examined according to standard quercetin and the results are given as mg quercetin equivalence per mg extract and per g sample.

This method is known as Zhishen method (1999) and applied in further researches with some adjustments (Zhishen, 1999). For the standard curve preparation, the steps are explained in appendix (Appendix A-6). The other required solutions used in this method are 5% $NaNO_2$, 10% $AlCl_3$, 1 M NaOH. For 5% $NaNO_2$ solution, 2.5 g $NaNO_2$ was weighed and put into a glass and then distilled water was added until being 50 ml. For 10% $AlCl_3$ solution, 5 g of AlCl3 was weighed and distilled water was added until being 50 ml. For 1 M NaOH solution, 2g NaOH was weighed and distilled water was added until being 50 ml.

At the beginning of the method, 0.5 ml of the serially diluted samples or serially diluted quercetin was piped into the tubes and 2 ml of distilled water added on it. At time 0 min, 0.15 ml of $NaNO_2$ solution was added on it. This mixture kept for 5min. At 5 min. 0.15 ml of 10% $AlCl_3$ solution was piped into the mixture. Then this mixture was kept for 6 min more, 1 ml of 1M NaOH was added on it. Then 1.2 ml of distilled water was piped out and added on it. The color was changed to pink after all these applications. The solutions were read by the spectrophotometer at 510 nm relative to the blank solution which was prepared by only with the solvent without any extract or quercetin stock solution addition. The mechanism behind this chemical reactions is based on the $AlCl_3$ catches the ketone group and hydroxyl group of the flavonoids to produce stable compounds with acids.

The quercetin results gives the absorbance values which are necessary for the preparation of the standard curve and the absorbance of the samples are calculated by the help of the quercetin standard curve. Figure 2.19 presents the chemical structure of quercetin.

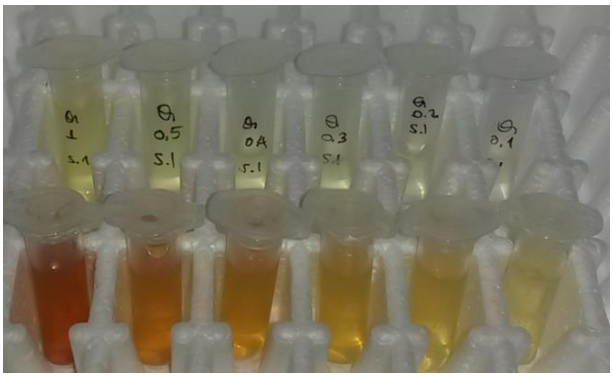


Figure 2.18 Serially diluted quercetin solutions for standard curve and the color change during the reaction

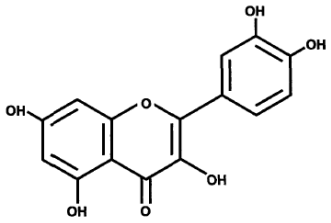


Figure 2.19 Chemical structure of quercetin

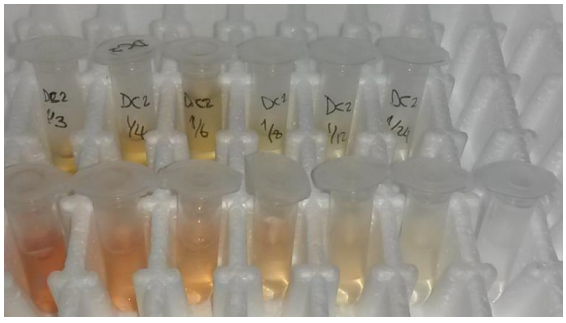


Figure 2.20 For DC2 sample, serially diluted extracts and different color changes after the method completed

Figure 2.18 shows the serially diluted quercetin solutions and their reaction and Figure 2.20 shows the serially diluted DC2 samples and the color change at the end of the reaction. According to the color changes the absorbance values were obtained and used for calculations in terms of quercetin equivalence values. The steps of the assay is given in the appendix part as steps (Appendix A-7)

2.4.5 Method of HPLC Analysis

Rapid reverse phase HPLC is used for chromatographic analysis. Basic principle of the HPLC method is related with the compound separation by using columns and solvents according to the polarities. The HPLC with C18 column (250 mm x 4.6 mm, 5 μm) was used for chromatographic separation. C18 column is nonpolar which catches the nonpolar molecules. 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 epicatechin and catechin, respectively. For the solvents binary mobile phase was 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 0,1%. Data was processed on an Intel pentium IV PC computer by using LC Solution Programme.

The solvent flow rates are important in order to catch the polar or non polar molecules. According to the method, polar solvent was given with higher ratio than nonpolar solvent. The flow rate started with 94% A solvent till 6^{th} minute. Then, A became 75% till 18^{th} min. For the next one minute, flow rate of A solvent became 40% and continued same until 21^{st} min. Then concentration of A increased with the same rate and the measurement stopped at 26^{th} min. This flow rate is explained with a table in appendix A-8 and the figure of HPLC is shown in appendix A-9.

The reason behind this flow rate is about polarity. If the first sent solvent is polar, then it sends the polar molecules at the beginning quickly. At the same time, nonpolar molecules were kept and went later and this provides to see on the graph nonpolar molecules later and more clearly. This method is applied for both the (+)-catechin and (-)-epicatechin standards and the samples.

(+)- Catechin and (-)-Epicatechin were dissolved and serially diluted in order to prodivde a standard curve. Then the same method is applied on the samples. In order to calculate the equivalent values of samples in terms of (-)-epicatechin and (+)-catechin, the area under the curves are used in the equation obtained from the standard curves. This area is related with the concentration and this found concentration explains the μg (+)-catechin or (-)-epicetechin / g sample.

CHAPTER 3

RESULTS AND DISCUSSION

In this research two different methods for antioxidant activity calculation are expressed. Total phenolic content was analyzed by using Folin-Ciocalteu Method. Total flavonoid content was expressed in terms of quercetin equivalence mg for mg extract in chocolates. HPLC is used for (-)-epicatechin and (+)-catechin amount detection. All the methods required for these analyses are expressed in the previous section. The content of this chapter is about the calculations, standard curves, detected amounts and their comparison with themselves and with the literature data.

3.1 Results of Defatting

Fat is a macronutrient and it is expressed on the labels of the food products. The chocolates used in this study have the labeling which shows the fat amount as a percentage in the nutrition data part.

Defatting procedure was applied on all milk and dark chocolate samples using n- hexane. The expected results which are given on the label of the chocolates are around 28-42% fat and the exact results measured for each sample are given below in Table 3.1

Table 3.1 The results of defatting for milk chocolate samples and dark chocolate samples

DEFATTING	MC1	MC2	MC3	DC1	DC2	DC3
Chocolate samples (g)	20.00	20.00	20.00	20.00	20.00	20.00
Defatted chocolate (g)	13.34	12.50	13.11	10.09	12.46	10.40
Defatted chocolate (g) in 100g sample	66.70	62.50	65.53	50.43	62.30	52.00

According to the table, after defatting by hexane, 50- 67% of the samples stays as the chocolate nonfat solid part. This result means around 33-50% of the samples were removed as the fat content. There is around 10% difference between expected and the real results and the reason of this difference is the loss experienced during the experiment. In conclusion, defatting results are quite close to expression on the nutritional information part on the label.

3.2 Results of Solvent Optimization for Extraction

Solvent optimization provides better efficiency in the analysis. It was applied on a dark chocolate sample with high cocoa content. Dark chocolate sample was dissolved in 3 different solvent and after drying the amount of extracts are given on the table below.

Table 3.2 % Yield amounts obtained for 3 different solvents.

	Solvent	Defatted Chocolate (g/100 g sample)	Extract (g/100 g sample)
S1	Acetone/Water/Acetic Acid (70:28:2 v/v/v)	49.57	19.46
S2	Acetone/Water (70:30 v/v)	49.57	20.09
S3	Distilled Water (100)	49.57	17.35

These 3 different extracts were prepared for further methods. The calculation details will be given for the next steps of this chapter for these methods. In this part, the results for DPPH, total phenol and total flavonoid methods are shown on the table below.

Table 3.3 Results for 3 different solvents for 3 different methods**.**

	S1	S2	S3
IC50 (DPPH)	0.31	0.35	0.76
GAE (mg/100 g DC) (Total phenol)	703	603	312
QE (mg/100 g DC) (Total flavonoid)	426	417	208

According to the table above S1 has the highest efficiency for three different method of antioxidant analysis. IC50 values give the required amount of mg/ml extract for 50% inhibition of DPPH ● free radical. If IC50 value is lower, it means less amount of chocolate is necessary to inhibition. For the total phenolic compound results, Gallic acid was used as the standard and the total phenolic compounds in the samples were calculated according to that standard curve. The highest detection is obtained in the extract prepared with solvent 1. For the other method, total flavonoid content determination, quercetin was used as the standard and according to quercetin standard curve, the flavonoid content in the dark chocolate was calculated. Even though it seems closer for the solvent 1 and 2 extracts, it is higher in the solvent 1. In addition, total phenolic compound are higher than the flavonoid compounds as expected. The main reason is that the flavonoids are the subgroup of the phenolic compounds.

These 3 samples are same. Then these differences mean that, extraction efficiency to get the highest amount of soluble antioxidant compounds is provided by the solvent1, acetone/water/acetic acid. One of the reasons behind this is related with the polarity of the solvents and according to Hui and Sherkat (2005) free radical scavenging activity is higher is nonpolar solvents for quercetin and epicatechin.

According to Adamson (1999), extraction for cocoa and chocolate polyphenols detection should be done by acetone / water / acetic acid. The results and the literature research supports to extraction with acetone / water / acetic acid solution (Cooper et al., 2007).

3.3 Results of Extraction

Extraction process was applied on the defatted samples. Acetone, water, acetic acid solution was used as the solvent for extraction. After drying the extract amount is calculated and the final concentrations of the extracts in solvents

were detected. These concentrations give the information about the extract in the solvent in terms of mg/ml.

In order to make further calculations about the samples, it is required to give also the extract amount obtained from 100 g sample.

Table 3.4 The concentration (mg extract / ml solution) of the 6 different samples.

Sample	Concentration of the extract (mg/ml)
MC1	71.97
MC2	71.78
MC3	73.13
DC1	58.47
DC2	75.57
DC3	63.98

Table 3.5 Extract amount in 100 g sample of 6 different samples

Sample	Defatted chocolate (g/100 g chocolate)	Extract (g/1 g defatted chocolate)	Extract in sample (g extract/100 g chocolate)
MC1	66.70	0.62	41.28
MC2	62.50	0.56	34.99
MC3	65.53	0.60	39.30
DC1	50.43	0.47	23.59
DC2	62.30	0.63	39.07
DC3	52.00	0.53	27.61

Extracts were applied serial dilutions in order to obtain different concentrations. These different concentrations were decided in order to provide the suitable range for the spectrophotometric and chromatographic

measurements and in order to provide these measurements in the standard curve range.

3.4 Results of Antioxidant Activity

3.4.1 Results of DPPH Free Radical Scavenging Activity

DPPH free radical scavenging activity determination analysis expresses the antioxidant capacity of the samples.

Extracts were applied serial dilution and DPPH free radical was dissolved in ethanol according to get the 1.4 abs at 517 nm. The abs values which were obtained during the spectrophotometric measurements were calculated as RSA%. The formulation which gives the RSA% is expressed below.

$$RSA\% = (A_0 - A_1) / A_0 * 100$$

The A_0 is the absorbance of the blank, which does not contain any extract but contains solvent. A_1 is the absorbance value of the extracts. The RSA% values were calculated according to 4 different measurements for each dilution of the each sample. Then this RSA% values were taken into account with the final concentration of the samples which were obtained after the completion of the DPPH and extract addition.

The graphs which are expressing the IC50 (half maximal inhibitory concentration) were drawn according to the final concentrations and the RSA% of the samples.

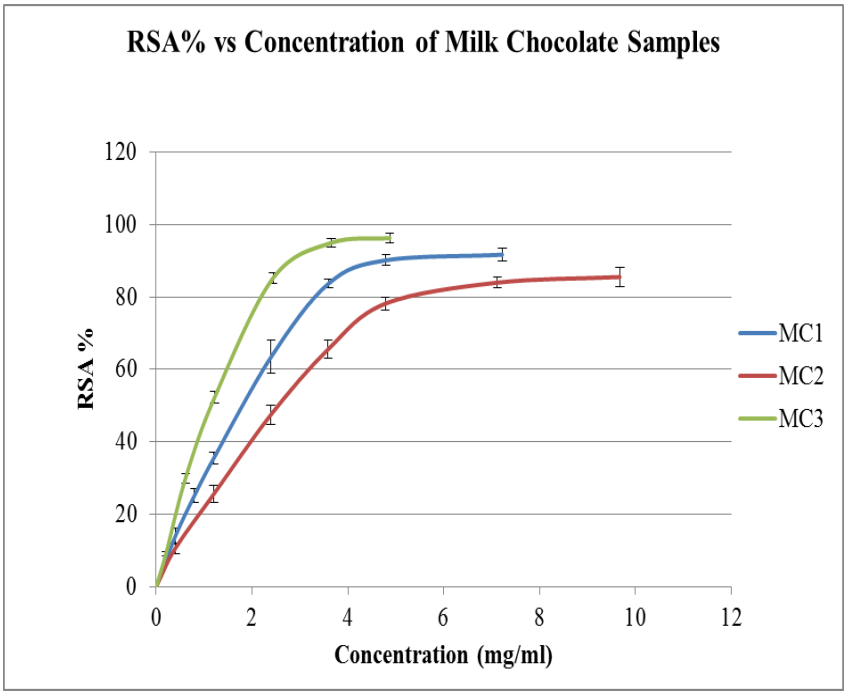


Figure 3.1 RSA% and the final concentration graph of MC samples

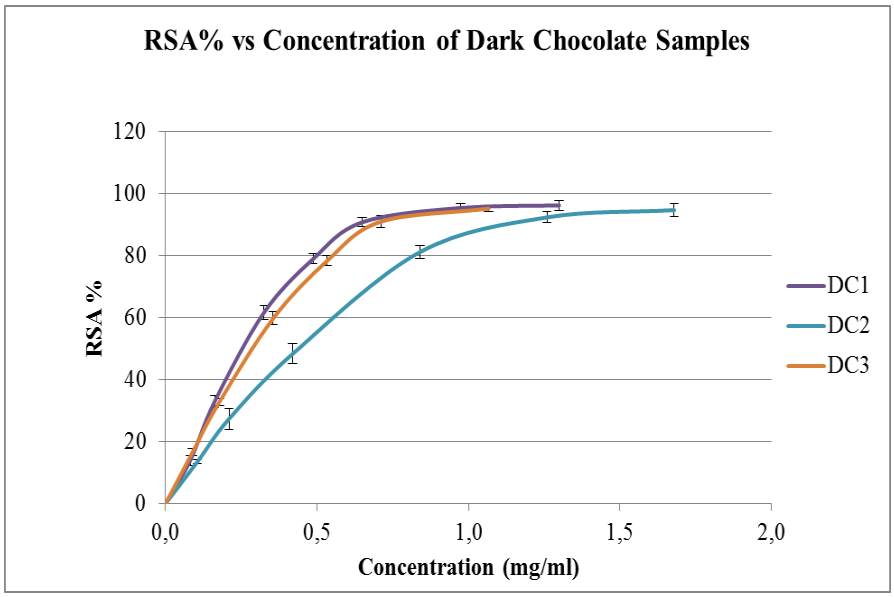


Figure 3.2 RSA % and the final concentration graph of DC samples

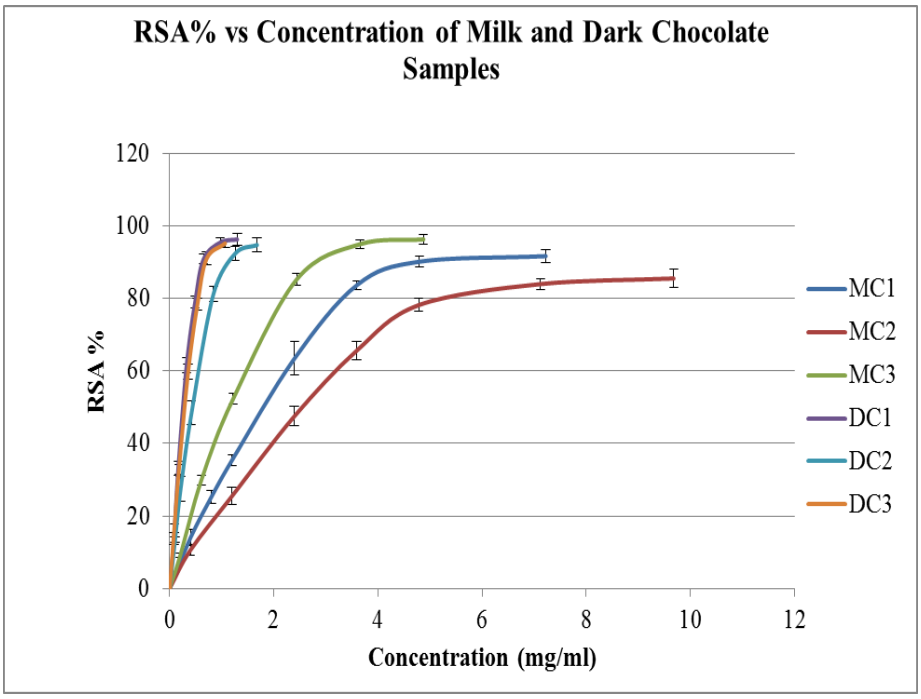


Figure 3.3 RSA% and the final concentration graph of MC and DC samples

According to the figures of RSA% and the final concentrations of the samples, the 50% radical scavenging activity occurs with smaller quantities of extracts for dark chocolates and higher for milk chocolates. Figure 3.2 indicates that the DC2 sample has lower antioxidant activity than the other 2 samples. For the milk chocolate samples, MC3 has the highest antioxidant activity and MC2 has the lowest. The RSA% and the final concentrations are in Appendix B with the RSA% information used for IC50 result calculation.

According to these results the IC50 values were calculated for all of the samples.

Table 3.6 IC50 values of 6 different samples with standard deviations

Sample	IC50 (mg extract/ml)	Sample	IC50 (mg extract/ml)
MC1	2.524±0.053	DC1	0.406±0.123
MC2	3.683±0.120	DC2	0.628±0.011
MC3	1.749±0.107	DC3	0.387±0.089

IC50 values were obtained from the RSA% and the final concentrations. IC50 values mean that the lower IC50 requires less amount of extract (mg/ml) to provide 50 percent inhibition of the DPPH free radical. Therefore lower IC 50 value indicates stronger antioxidant activity

In literature, IC values for milk chocolate is higher than the dark chocolates. IC50 values should be considered according to not only the extract amounts but also the sample amounts in order to provide more accurate comparison.

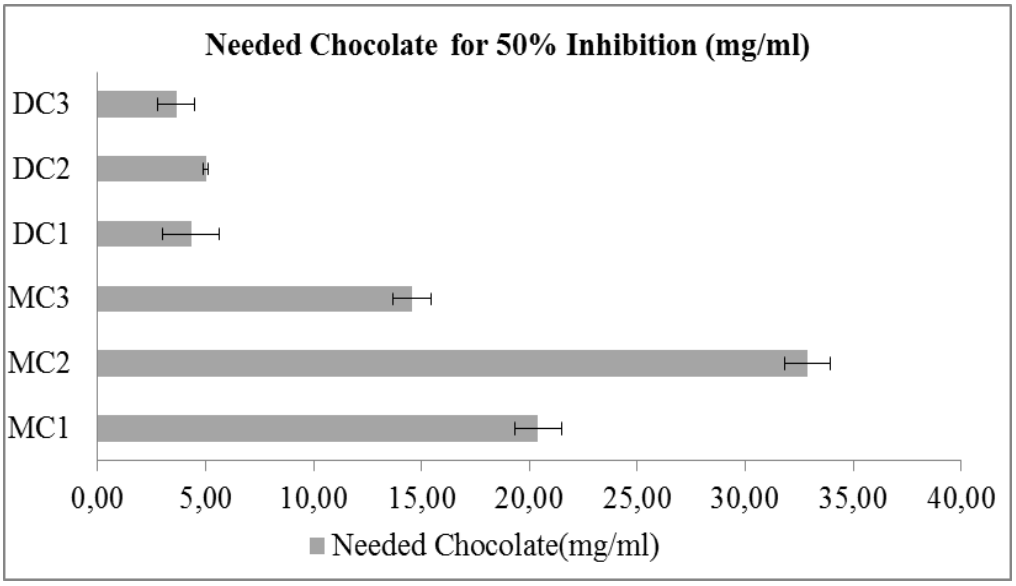


Figure 3.4 Sample amounts required to provide 50 % inhibition of DPPH free radical

Table 3.7 Needed chocolate sample amounts for 50% inhibition

Sample	Needed Chocolate(mg/ml)	STD Deviation
MC1	20.39	1.1
MC2	32.89	1.1
MC3	14.58	0.9
DC1	4.34	1.3
DC2	5.01	0.1
DC3	3.64	0.8

This table expresses the difference between the samples in terms of required same antioxidant effect. Higher the amount of samples, lower the antioxidant content. The MC2 has the lowest antioxidant capacity and DC3 has the highest antioxidant capacity. According to the results, in order to provide same antioxidant effect, the required amount of milk chocolate is 22.62±1.01 mg/ml and for dark chocolate this amount is 4.33±0.75 mg/ml. Dark chocolate provides more antioxidant activity with more polyphenol content.

3.4.2 Results of ABTS Free Radical Scavenging Activity

The antioxidant activity of cocoa and cocoa cake samples were measured by ABTS method and in terms of Trolox Equivalent (TE). At the beginning the Trolox standard was used to inhibit ABTS solution and to form standard curve. The linear line equation y = 0,0422x gives the relation between μM Trolox concentration and % inhibition. Trolox standard curve values are given in the appendix B.

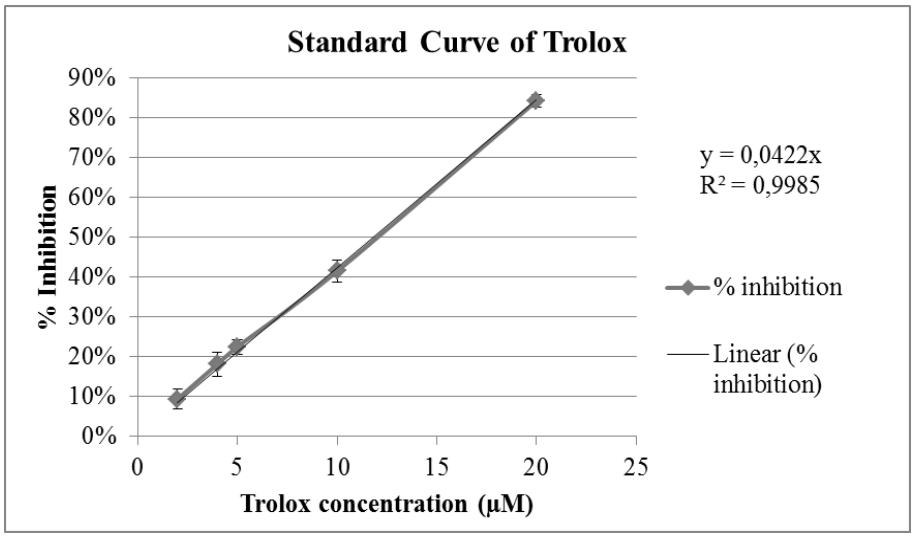


Figure 3.5 Standard curve for Trolox

The samples were applied the same procedure and they were measured 4 times. % Inhibition value for all the samples was calculated with the absorbance values. By using this abs values and the equation in the Trolox standard curve, the TE of the samples have been calculated.

According to the results the milk chocolates has average 29.21±2.57 µmol TE/g sample and for dark chocolates 144.09±16.56 µmol TE/g sample. This result is parallel with the DPPH● results, as expected.

Table 3.8 TEAC results for g extracts of the 6 different samples

Sample	µmol TE/g extract	STD Deviation
MC1	22.35	1.20
MC2	19.26	1.58
MC3	46.04	4.92
DC1	163.37	19.19
DC2	109.03	10.80
DC3	159.89	19.68

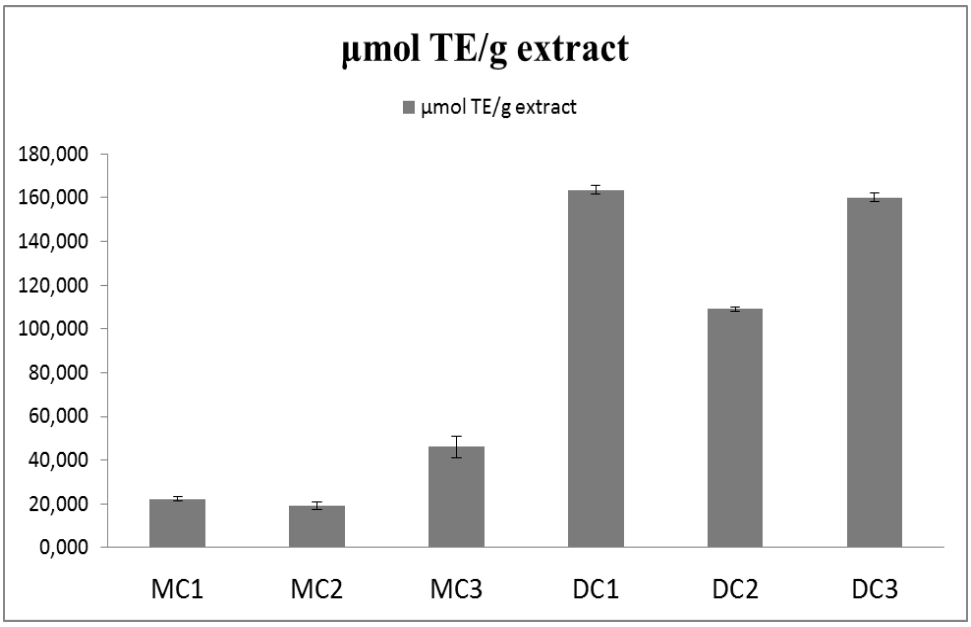


Figure 3.6 TEAC results comparison for g extracts of the 6 different samples

When Figure 3.6 is considered, the results show that there are variations in the TEAC equivalents results of the extracts; however the results of dark chocolates and milk chocolate are closer. Milk chocolates have around 30% cocoa contents and there is almost two times higher results for MC3 chocolate according to the others. According to Gu et al. (2006) milk chocolates have around 80 µmol TE/g sample and dark chocolates have 228 µmol TE/g sample. Although there is difference between the results they are similar to each other and the results of this study also parallel with high TE value for dark chocolate than the milk chocolate. The reason of the variations between similar cocoa containing ones should be dependent on the cocoa type or cocoa processing conditions of the chocolate production steps.

3.5 Results of Total Phenol Content

Total phenolic content is analyzed and calculated according to the Folin-Ciocalteu Method.

Gallic acid as the standard was analyzed in order to obtain the standard curve. This standard curve was drawn according to the final concentration of the Gallic acid and absorbance values that it gave at 750 nm.

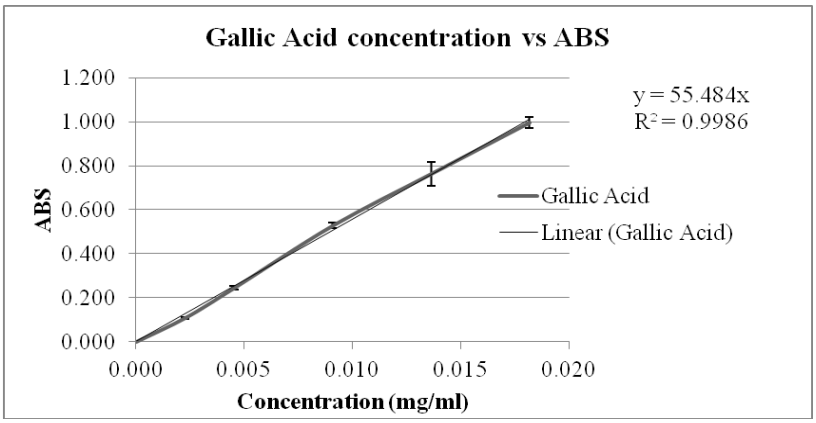


Figure 3.7 Gallic acid standard curve for the Folin-Ciocalteu Method

According to this standard curve the linear equation was obtained. This equation was used for further calculations as the y value is the absorbance and the x value gives the concentration of the Gallic acid at that absorbance, when the x value is divided by the final concentration of the extract, the result will give the Gallic acid equivalence of the sample. The table below gives the results of these calculations for all of the samples.

Table 3.9 GAE values of the 6 different chocolate samples**.**

TPC	GAE (mg/ mg extract)	STD Dev	GAE of chocolate (mg/100 g sample)	STD Dev
MC1	0.007	0.001	**276.55**	24.01
MC2	0.005	0.001	**172.14**	14.99
MC3	0.010	0.001	**398.77**	8.22
DC1	0.042	0.003	**995.37**	63.46
DC2	0.024	0.002	**922.34**	89.93
DC3	0.040	0.003	**1113.01**	76.66

When the Gallic acid equivalence values are compared there is high difference between the dark chocolate and the milk chocolate which is expected. Milk chocolates average value is 282.48±15.74 mg GAE/100 g and for dark chocolate 1010.24±76.68 mg GAE/100 g. However, there is also difference between the milk chocolates it is not only because of the cocoa content amount but also from the cocoa quality and the processing conditions of the chocolate and the cocoa production.

According to Wollgast et al. (2000) other researches the dark chocolate total polyphenols amount is around 800 mg GAE per 100 g. This is very close to the result obtained from dark chocolate samples. The milk chocolate polyphenols content is given around 500 GAE mg per 100 g sample. MC 1 is close to the research results; on the other hand MC2 is less than the expected. On the other hand, Alderton et al. (2014) milk chocolates which is used for cardiovascular diseases researches contains around 230 mg total polyphenols per 100 g sample. This is quite close to the MC sample data. The difference between the samples is supported by the DPPH results. This sample, MC 2 has the highest IC50 value which means the antioxidant content is less than the other 2 milk

chocolate samples. MC3 and dark chocolates' total phenol content results are close to the result obtained from literature research.

3.6 Results of Total Flavonoids Content

Total flavonoid content is analyzed and calculated according to the methods given in the material method chapter. Quercetin was used as the standard. The standard curve for the further measurements was obtained by the serially diluted quercetin solutions. All the measurements were repeated 4 times for both the quercetin and the samples. The standard curve was drawn by the final concentrations and the absorbance values at 510 nm.

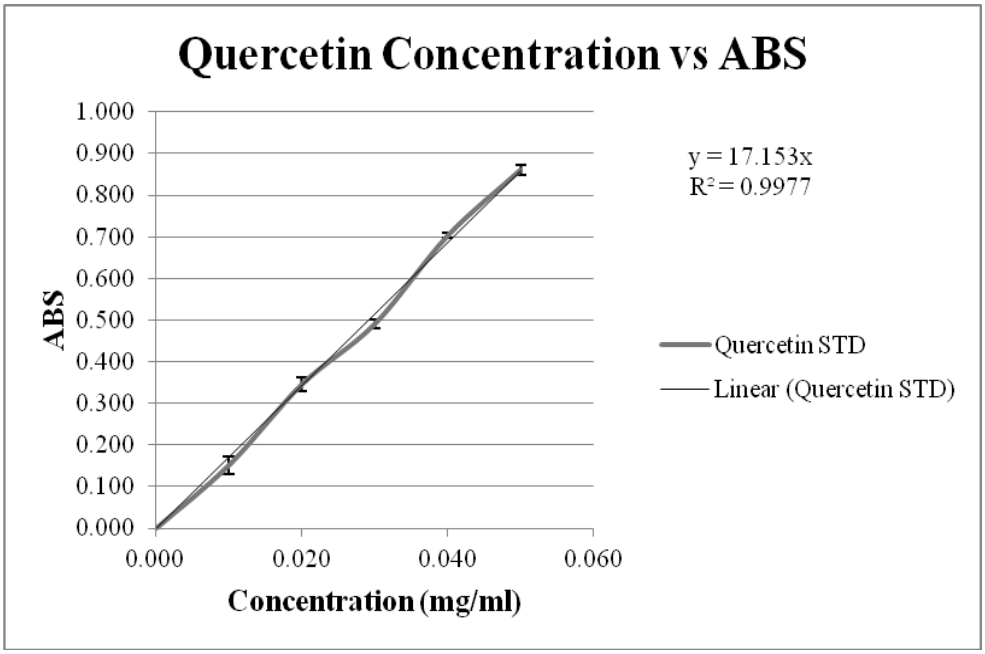


Figure 3.8 Quercetin standard curve and the equation

According to Figure 3.8, the linearization equation is obtained and the y values were the absorbance values of the samples, then the x values are obtained as the Quercetin concentration at that absorbance. When this x values were divided by the final concentrations of the samples, the results gave the Quercetin equivalence value of the samples as mg per mg extract.

In order to make better comparison, it is required to make the total flavonoid content of 100 g sample in order to make more accurate comparison.

Table 3.10 Total flavonoid content of the 6 different chocolate samples

TFC	QE (mg/ mg extract)	STD Dev	QE (mg/100 g sample)	STD Dev
MC1	0.004	0.001	165.03	5.13
MC2	0.003	0.001	89.30	5.54
MC3	0.006	0.001	251.67	17.00
DC1	0.025	0.002	590.42	46.84
DC2	0.012	0.001	473.82	48.91
DC3	0.023	0.002	642.18	49.88

According to the Table 3.8 the total flavonoid contents of the chocolate samples are higher for dark chocolates than the milk chocolates. The average TFC for milk chocolate is 168.67±9.22 mg / 100 g sample and for dark chocolate 568.81±48.54 mg/100 g sample. This analysis result also indicates that the MC2 sample has the lowest level of the total flavonoid content in terms of QE. When these values are compared with the total polyphenols content, they are less than the total polyphenols content of the samples.

According to the Fernanda et al. (2009), the total flavonoids are the 34% of the total polyphenols. This value is 21% for the milk chocolates. When the results

are compared the ratios between the samples can be explained with the total procyanidin content.

1.6.1 Comparison of TPC and TFC assays

Flavonoids are one subgroup of polyphenols and total flavonoid content of a food is expected to be less than the total phenol content.

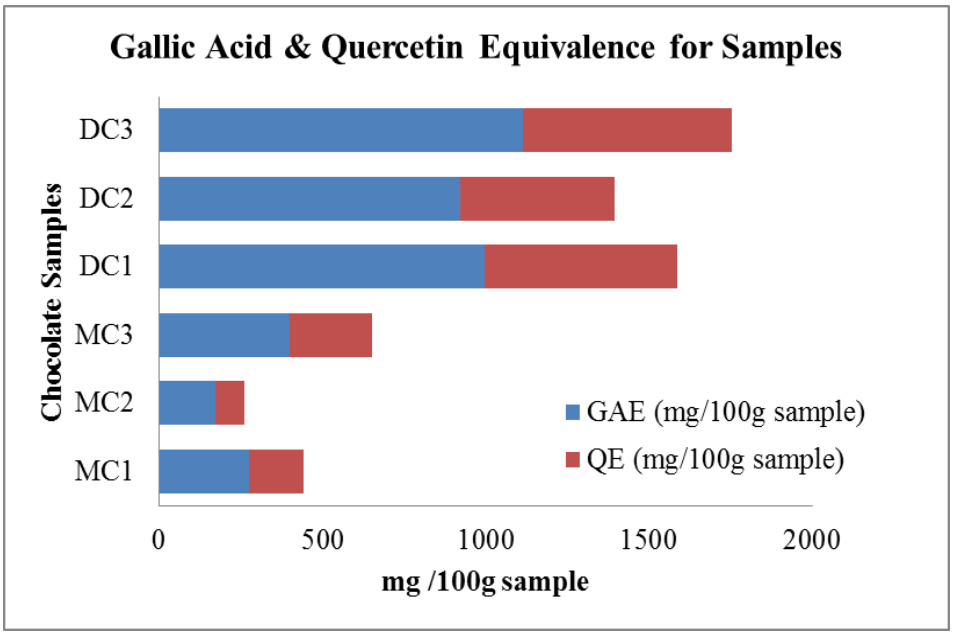


Figure 3.9 GAE and QE comparison for 6 different samples

According to the Figure3.9, the flavonoid content is the significant part of phenolic content of chocolates. The main reason is the (-)-epicatechin and (+)-catechin content of the chocolates. These antioxidants are two of the most important phenolic contents of chocolates.

3.7 Results of HPLC

The HPLC analysis was applied to all the samples. The standards were (-)-epicatechin and (+)-catechin. They are the most important antioxidants which are found in chocolates. Epicatechin is eluted from the column at 23.6+/-0.1 min. Catechin is eluted at 15.6±0.1 min. (-)-Epicatechin and (+)-catechin standards were measured from serially diluted solutions in order to obtain different area values for different concentrations and to obtain a standard curve for both of them. The standard curve gave the equation for samples to calculate as (-)-epicatechin and (+)-catechin equivalence values.

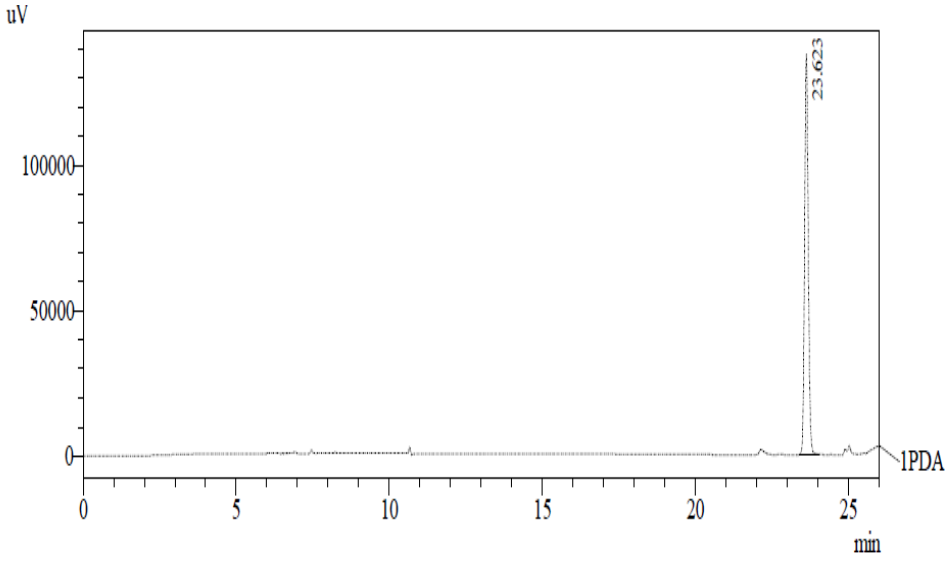


Figure 3.10 (-)-Epicatechin standard peak on reversed phase HPLC.

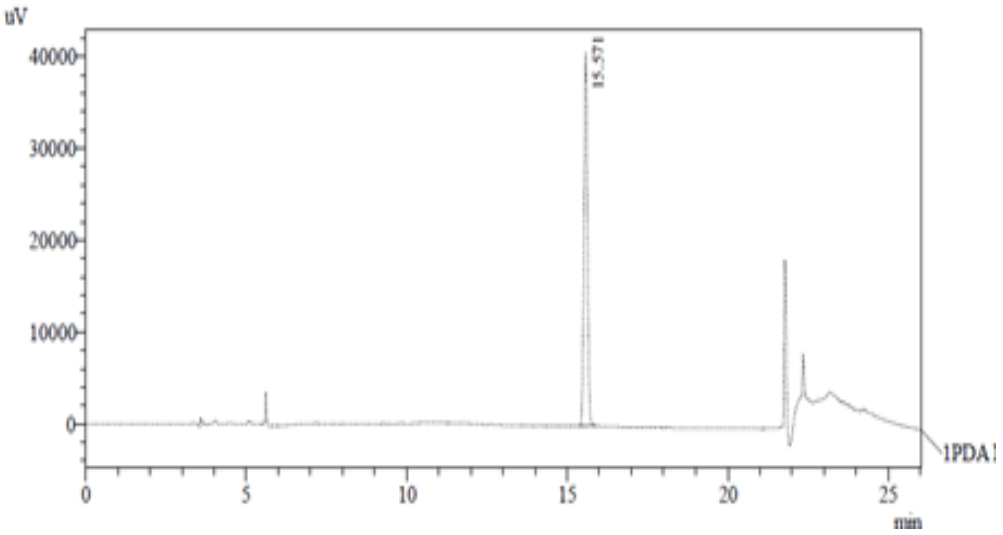


Figure 3.11 (+)-Catechin standard peak on reversed phase HPLC

According to these standard peaks, the samples graphs were analyzed. The area under the curve which comes at the same time with the standard gives the (-)-epicatechin or (+)-catechin values for the samples. For the calculations from area to concentration in the extract, the standard curve for the standards was used separately.

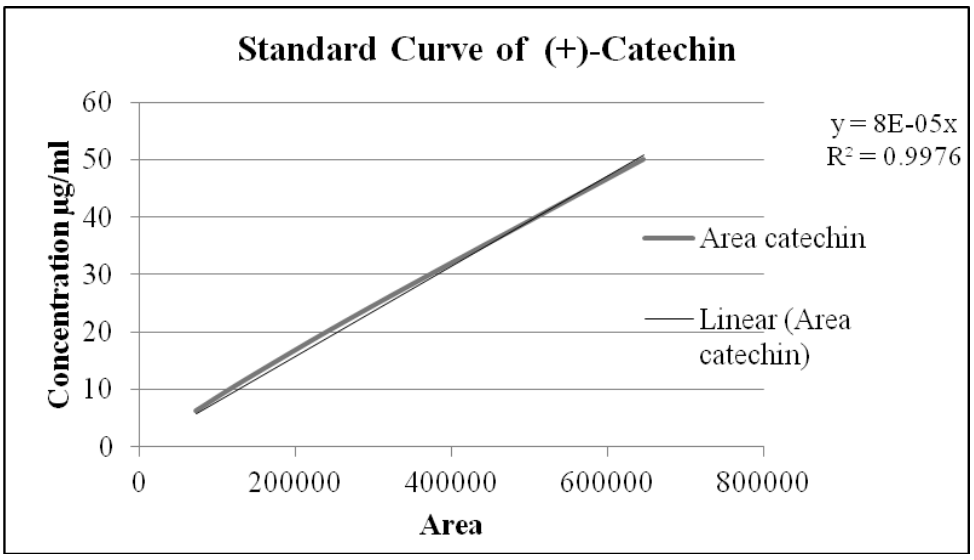


Figure 3.12 Standard curve for (+)-catechin and the equations for calculations of the samples.

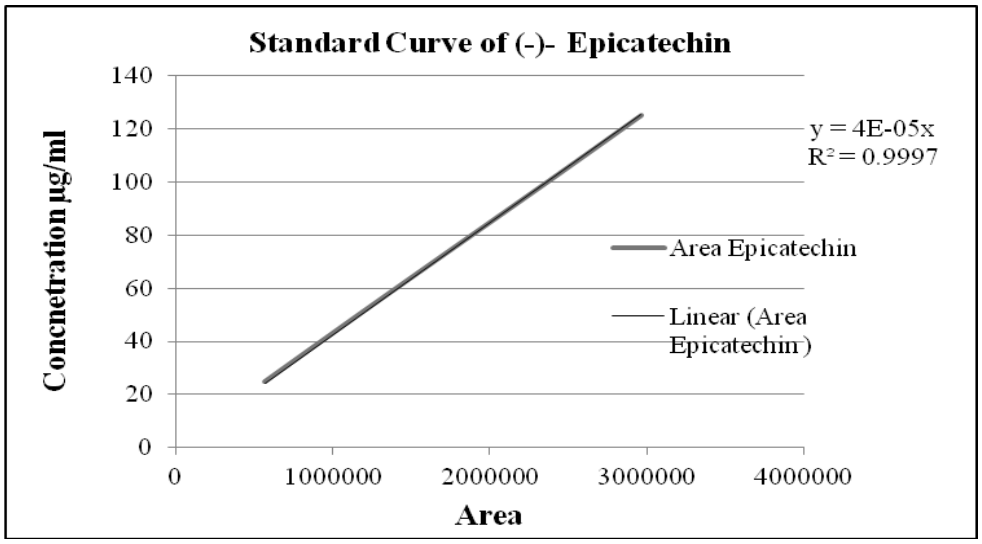


Figure 3.13 Standard curve for (-)-epicatechin and the equations for calculations of the samples.

These standard curves were obtained by the measurements made from serially diluted (+)-catechin and (-)-epicatechin solutions. The equations on the standard curves were used for the calculations of the samples. Samples were diluted and filtered in order to obtain them in the range of standard curve. The areas under the curves which were obtained from the HPLC analysis are shown in Figure 3.14.

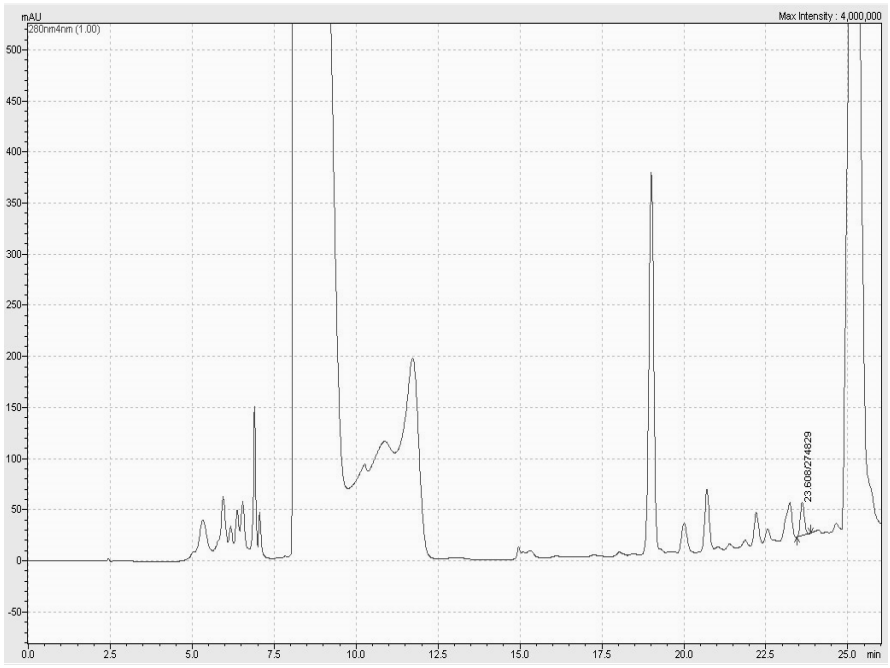


Figure 3.14 (-)-Epicatechin peak for reversed phase HPLC of DC2

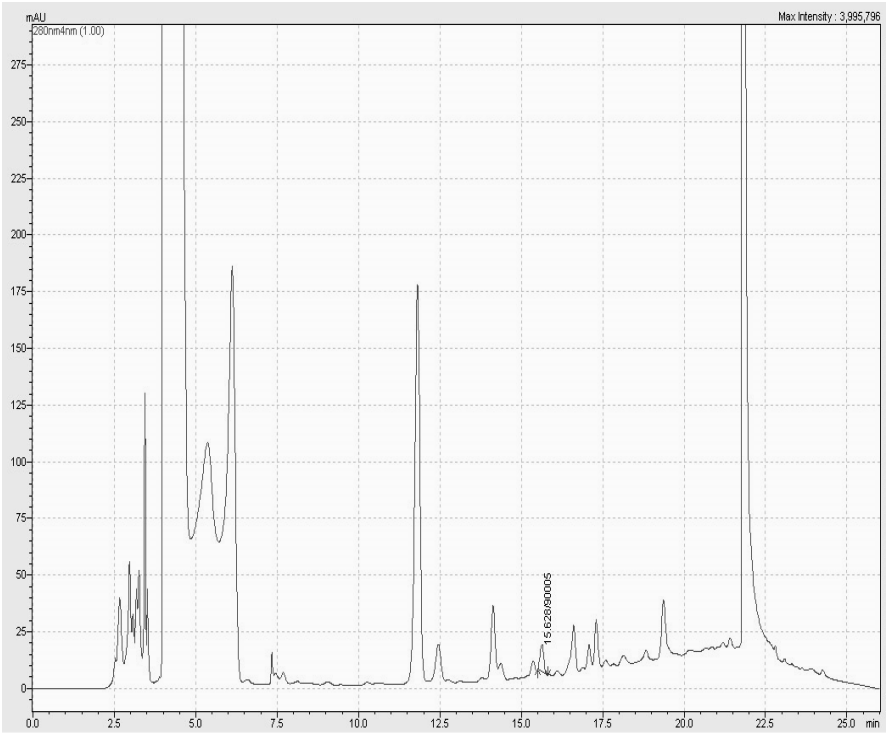


Figure 3.15 (+)-Catechin peak for reversed phase HPLC for DC2

The values of catechin and epicatechin amounts obtained from HPLC analysis are expressed in the Table 3.11. The calculation was done with the area under curve values of the extracts at 280 nm according to the equation of the standard curve.

Table 3.11 (-)-Epicatechin content of samples

Sample	µg (-)-Epicatechin/mg extract	mg (-)-Epicatechin/g chocolate	mg (-)-Epicatechin/100g chocolate
MC1	0.147	0.061	6.05
MC2	0.066	0.023	2.32
MC3	0.314	0.123	12.34
DC1	1.058	0.249	24.95
DC2	0.343	0.134	13.42
DC3	0.826	0.228	22.82

According to the results, it has been found that the milk chocolates have lower (-)-epicatechin values than for dark chocolates. The average of the milk chocolate (-)-epicatechin amount is 0.069 ± 0.050 mg EC /g chocolate and for dark chocolate this value is 0.192 ± 0.081 mg EC /g chocolate.

Table 3.12 (+)- Catechin content of samples

Sample	µg (+)-Catechin/mg extract	mg (+)-Catechin/g chocolate	mg (+)-Catechin/100g chocolate
MC1	0.304	0.125	12.54
MC2	0.168	0.059	5.88
MC3	0.221	0.087	8.68
DC1	1.043	0.246	24.61
DC2	0.448	0.175	17.50
DC3	0.872	0.241	24.08

According to the results, it has been found that the milk chocolates have lower (+)-catechin values than for dark chocolates. The average of the milk chocolate (+)-catechin amount is 0.090 ± 0.033 mg C /g chocolate sample and for dark chocolate this value is 0.221 ± 0.040 mg C /g chocolate sample.

Table 3.13 Total (-)-epicatechin and (+)-catechin content of chocolate samples

Sample	Total epicatechin and catechin mg/100 g chocolate
MC1	18.59
MC2	8.20
MC3	21.02
DC1	49.55
DC2	30.92
DC3	46.90

According to the results, it has been found that the milk chocolates have lower (+)-catechin and (-)-epicatechin values than for dark chocolates. The average of the milk chocolate (+)-catechin and (-)-epicatechin amount is 0.159 ± 0.070

mg /g chocolate sample and for dark chocolate this value is 0.425 ± 0.101 mg /g chocolate sample. For both assay DC1 and DC3 are so close to each other and the MC2 has the lowest amount of EC and C equivalence values.

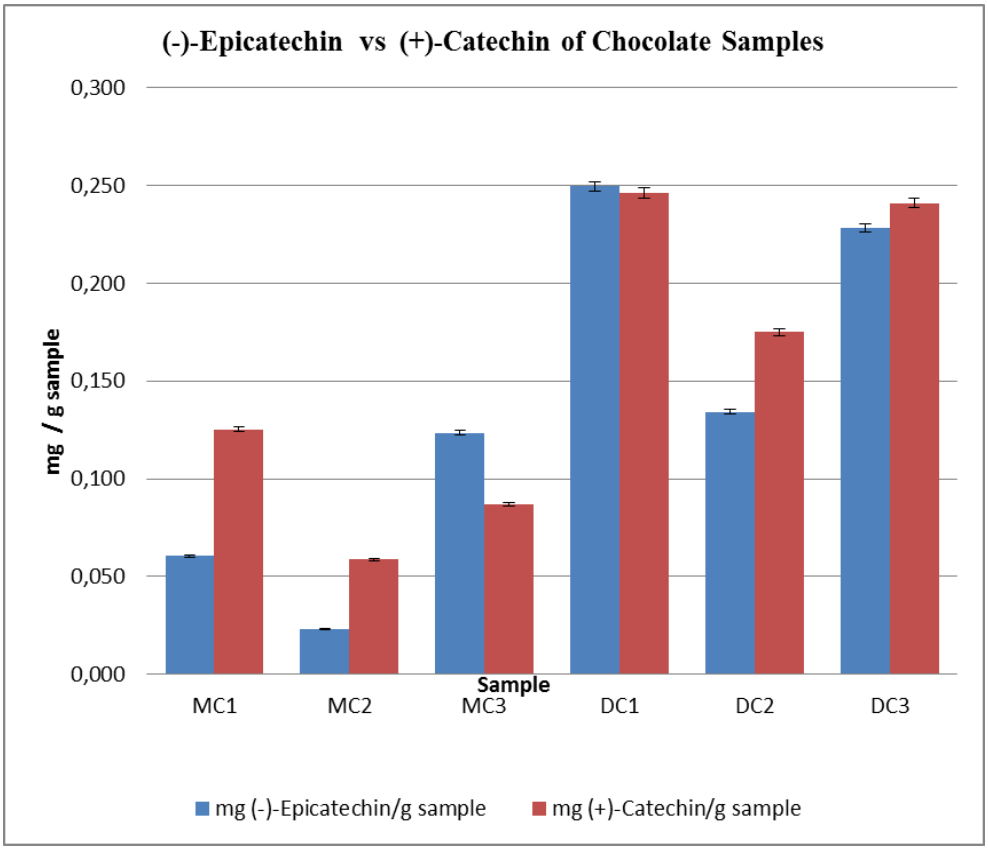


Figure 3.16 EC and C values for all samples as mg/g sample

As it shown in Figure 3-12, dark chocolates have higher amount of EC and C contents. According to milk and dark chocolates should be between 0.18-1.25 mg/g sample and C of them should be between 0.043-1.519 mg/g sample and the results of current study is in the reference range existing in literature. In another research, there were 3 different DC and 3 different MC samples analyzed for their phenolic compounds. The comparison table is given below.

Table 3.14 Comparison of the results with literature

	EC mg/g sample		**C mg/g sample**		**EC&C mg/g sample**	
	Current Study	**Similar reference research**	**Current Study**	**Similar reference research**	**Current Study**	**Similar reference research**
DC1	0.249	0.326	0.246	0.151	0.496	0.478
DC2	0.134	0.312	0.175	0.107	0.309	0.419
DC3	0.228	0.371	0.241	0.233	0.469	0.605
MC1	0.061	0.023	0.125	0.006	0.186	0.029
MC2	0.023	0.126	0.059	0.041	0.082	0.167
MC3	0.123	0.148	0.087	0.082	0.210	0.231

Results of HPLC analysis of this study is quite close to the literature data and some differences between the results and the references exist. There are other phenolic compounds in chocolate which come from cocoa content of it. According to Gu et al. (2009), the other important antioxidant molecules in chocolates are monomers, dimers, trimmers, trimers and tetramers. The cocoa used for all of the chocolates is the most important indicator of the results which directly depend on the cocoa type and quality, and its production conditions. (Gu et. al., 2009)

In another research made in Malasian market for commercially available chocolates, the results are a bit different. The dark chocolates have 274.35±1.40 mg EC /100 g sample and 184.80±1.14 mg C / 100 g sample. This result is almost 10 times higher than Turkish market results and this shows that the cocoa used for chocolate production can directly affect the phenolics in chocolates. However, for the milk chocolate, the results are not higher than this study's results. According to Malasian Market research, the lowest detectible limit is 0.03 mg/mL sample for EC and 0.01 mg/mL sample for C.

(-)-Epicatechin amount differs and it is converted to other polyphenols with processing conditions. For (-)-epicatechin, it is easy to be converted to (+)-catechin compound which is more stable than EC. All the industrial chocolates are produced by using processed cocoa and during all the steps of cocoa production, especially during fermentation, roasting and alkalization, the phenolic compounds are damaged.

Even though all the processing steps, chocolates which are commercially available in Turkish market, has close results according to other literature researches made for different commercially available chocolates found in different countries.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

In this research chocolates in the Turkish market have been analyzed in terms of antioxidant capacity and phenolic compound content. Chocolate is known with its beneficial impacts on the human health especially because of its high (-)-epicatechin and (+)-catechin content.

This study explains the conditions of the chocolate which are commercially available and common in Turkish market. Dark chocolates and milk chocolates have been selected for the research because of their high cocoa content according to white chocolates. In order to provide more accurate results, four different analytical method and HPLC method of analysis have been applied on the three different milk chocolate and three different dark chocolate samples. Milk chocolate samples have almost the same cocoa and milk contents and only DC2 has lower cocoa content than DC2 and DC3 which have similar ones. The results are parallel with the cocoa content of the samples. However, for the milk chocolates, results are around 30-50% lower than the other milk chocolates. This is an indicator for that the only impact of the phenolic content of the chocolates is not their cocoa level. The cocoa origin, quality, and process conditions are the significant factors. In addition, the milk content of the chocolate lowers the polyphenol content and antioxidant activity of cocoa polyphenols.

The antioxidant activity results made by ABTS● and DPPH● analysis and TPC and TFC analysis confirm each other. For HPLC analysis the EC and C contents of Turkish market chocolate are close to the literature.

In addition, (-)-epicatechin content of dark chocolates are also significantly high. For normal and healthy diet, adult people take 1000 mg polyphenols from foods that they consume. If 1 portion of dark chocolate is accepted as 40g, this will provide around 40% of polyphenol daily intake. This is the result of 70-80% cocoa containing dark chocolates. The (-)-epicatechin amount is high in the high cocoa containing samples and this brings many health benefits of EC also. As mentioned in previous chapters, daily intake of EC helps lowering blood pressure, reducing cancer risk, preventing lipid peroxidation, improving brain health, reducing oxidative stress.

In conclusion, chocolates commercially available in Turkish market have good antioxidant capacity according to the other worldwide researches. However, there is high difference between dark chocolates and milk chocolates in terms of antioxidant capacity. By consuming high cocoa containing dark chocolates, antioxidant intake increases significantly. Recommendations can be about producing more health beneficial chocolates with additional cocoa or antioxidant content. These products can be sold with functional health benefit claims. In addition the cocoa processing condition can be improved in order to protect the phenolic compounds.

REFERENCES

Adamson, G. E., Lazarus, S. A., Mitchell, A. E., Prior, R. L., Cao, G., Jacobs, P. H., ... & Schmitz, H. H. (1999). HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. Journal of Agricultural and Food Chemistry, 47(10), 4184-4188

Afoakwa, E. O. (2010). *Chocolate science and technology*. Oxford, U. K.: Wiley-Blackwell.

Aisbitt, B. (2008). Chocolate: the dark side? *British Nutrition Foundation Nutrition Bulletin*, 33, 114–116.

Ali, F., Ismail, A., & Kersten, S. (2014). Molecular mechanisms underlying the potential antiobesity-related diseases effect of cocoa polyphenols. *Molecular Nutrition & Food Research, 58*, 33–48.

Andujar, I., Recio, M. C., Giner, R. M., & Ríos, J. L. (2012). Cocoa polyphenols and their potential benefits for human health. Oxidative medicine and cellular longevity, pp.1-23. doi:10.1155/2012/906252

Arnao, M. B., Cano, A., & Acosta, M. (2001). The hydrophilic and lipophilic contribution to total antioxidant activity. Food Chemistry, 73(2), 239-244.

Beckett, S. T. (2008). *The science of chocolate*. York, UK: RSC Publishing.

Blois, M.S. 1958. Antioxidant determinations by the use of a stable free radical. Nature, 181, 1199-1200. doi:10.1038/1811199a0

Brand-Williams, W., Cuvelier, M. E., & Berset, C. L. W. T. (1995). Use of a free radical method to evaluate antioxidant activity. LWT-Food Science and Technology, 28(1), 25-30

Bravo, L. (1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews, 56*(11), 317-333.

Craft, B. D., Kerrihard, A. L., Amarowicz, R., & Pegg, R. B. (2012). Phenol-based antioxidants and the In Vitro Methods used for their assessment. *Comprehensive Reviews in Food Science and Food Safety, 11*, 148-173.

Dixon, R.A., Xie, D., & Sharma, S. B. (2005). Proanthocyanidins – a final frontier in flavonoid research? *New Phytologist, 165*, 9–28.

Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. Clarendon Press, Oxford, 1989.

Haminiuk, C. W. I., Maciel, G. M., Plata-Oviedo, M. S. V., & Peralta, R. M. (2012). Phenolic compounds in fruits – an overview. *International Journal of Food Science and Technology, 47*, 2023–2044.

Latif, R. (2013). Chocolate/cocoa and human health: a review. *The Journal of Medicine, 71*(2), 63-68.

Lee, K. W., Kim, Y. J., Lee, H. J., & Lee, C. Y. (2003). Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. *Journal of Agricultural and Food Chemistry, 51*, 7292-7295.

Lee, K. W., Kim, Y. J., Lee, H. J., & Lee, C. Y. (2003). Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. Journal of Agricultural and Food Chemistry, 51, 7292-7295.

López-Alarcón, C., Speisky, H., & Lissi, E. (2007). Antioxidant effect of 5-amino salicylic acid on copper-mediated LDL oxidation. *Biological Research, 40*(2), 155-162.

Martínez, R., Torres, P., Meneses, M. A., Figueroa, J. G., Pérez-Álvarez, J. A., & Viuda-Martos, M. (2012). Chemical, technological and in vitro antioxidant properties of cocoa (Theobroma cacao L.) co-products. Food Research International, 49(1), 39-45.

Patton, C. L., Ford, L. P., & Crunkleton, D. W. (2005). *Welcome to ChE: Chocolate engineering*. Paper presented at American Institute of Chemical Engineers Annual Meeting, Cincinnati, OH, October 30 - November 4.

Pérez-Jiménez, J., Neveu, V., Vos, F., & Scalbert, A. (2010). Identification of the 100 richest dietary sources of polyphenols: an application of the Phenol-Explorer database. European journal of clinical nutrition, 64, S112-S120. doi: 10.1038/ejcn.2010.221

Sagdicoglu Celep G, Marotta F. Oxidants and antioxidants in health and disease. *Oxid Antioxid Med Sci 2014; 3(1):x-x (ISSN: 2146-8389)*

Scalbert, A., Johnson, I. T., & Saltmarsh, M. (2005). Polyphenols: antioxidants and beyond. The American journal of clinical nutrition, 81(1), 215S-217S.

Schenker, S. (2000). The nutritional and physiological properties of chocolate. *British Nutrition Foundation Nutrition Bulletin, 25*, 303–313.

Schinella, G., Mosca, S., Cienfuegos-Jovellanos, E., Pasamar, M., Muguerza, B., Ramón, D., & Ríos, J. (2010). Antioxidant properties of polyphenol-rich cocoa products industrially processed. Food Research International, 43(6), 16141623

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture, 16, 144–153.

Stahl, L., Miller, K. B., Apgar, J., Sweigart, D. S., Stuart, D. A., McHale, N., ... & Hurst, W. J. (2009). Preservation of cocoa antioxidant activity, total polyphenols, flavan-3-ols, and procyanidin content in foods prepared with cocoa powder. Journal of food science, 74(6), C456-C461. doi: 10.1111/j.1750-3841.2009.01226.x.

Weichselbaum, E., & Buttriss, J. L. (2010). Polyphenols in the diet. British *Nutrition Foundation Nutrition Bulletin, 35*, 157–164.

World Healt Organization (n.d.). Retrieved October 28, 2014 from http://www.who.int/mediacentre/factsheets/fs310/en/

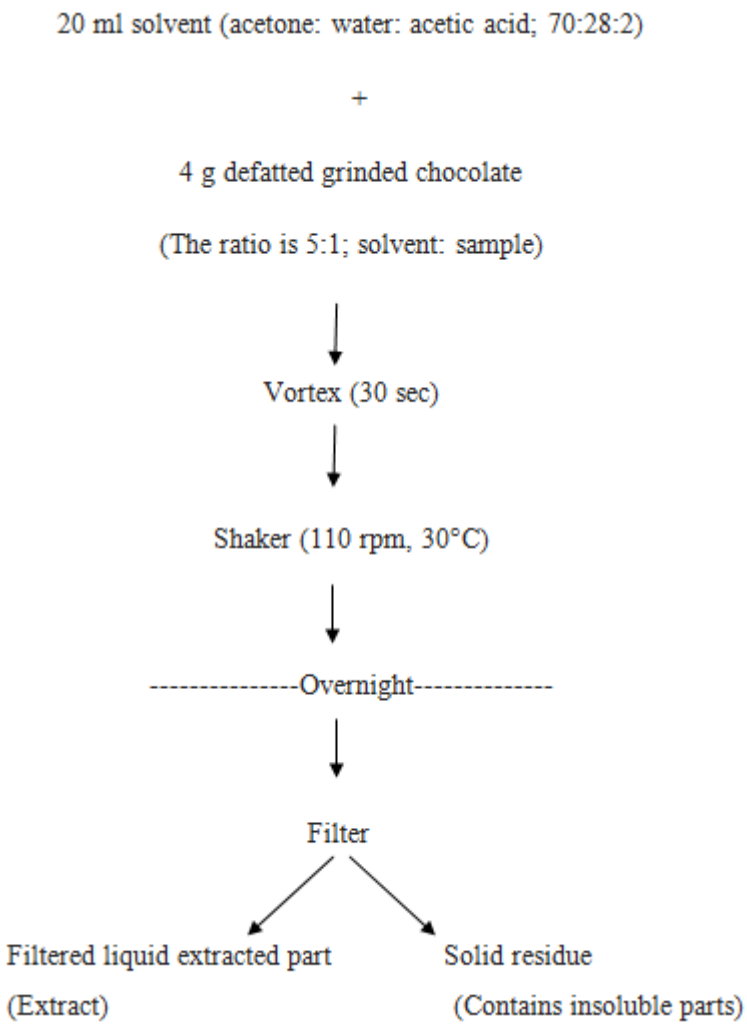
Zhishen, J., Mengcheng, T. and Jianming, W. 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chemistry, 64, 555−559

Zumbe, A. (1998). Polyphenols in cocoa: Are there health benefits? British *Nutrition Foundation Nutrition Bulletin, 23*, 94–102.

APPENDIX

Appendix A. Flow Charts of Methods

Appendix A-1 Flowchart for the extraction process



Appendix A-2 Steps of ABTS free radical stock solution preparation steps

ABTS stock solution preparation:

2,45 mM $K_2S_2O_8$ + 7 mM ABTS powder

= Mix and kept at dark for 16 hr

= ABTS stock solution

ABTS stock solution + Methanol

(Dilution until 0,70 +/- abs at 734 nm on spectrophotometer)

=

Ready for analysis and should be kept always in dark

Appendix A-3 Steps of ABTS free radical scavenging activity assay

ABTS Free radical scavenging activity measurement method:

10 µl Trolox or extract + 1000 µl ABTS solution

= Vortex (30 sec)

= Store in dark for 6 min at 30°C

=

Read by spectrophotometer at 734 nm

Appendix A-4 Steps for Gallic acid standard stock solution preparation

Standard stock solution preparation:

20 mg Gallic acid powder + 50 ml solvent (acetone/water/acetic acid)

= Gallic acid stock solution (0.4 mg GA/ml solution)

Dilution (0.4, 0.3, 0.2, 0.1, 0.05 mg/ml)

Appendix A-5 Steps for TPC assay

Steps for method:

0.15 ml extract or Gallic acid solution

+ 3 ml 2% Na_2CO_3 solution

= Vortex (for 15 sec)

= Kept at dark for 3 min

+

0.15 ml 50% Folin Reagent

= Vortex (for 15 sec)

=Kept at dark for 30 min

=Read at 750 nm by spectrophotometer relatively to blank

Appendix A-6 Quercetin Standard solution preparation for TFC assay

- For the standard curve, quercetin which is normally found in powdered form was dissolved in the extraction solvent
- Extraction solvent was acetone/water/acetic acid for this study.
- The concentration was arranged as 1, 0.5, 0.4, 0.3, 0.2, 0.1 mg quercetin/ml solvent.

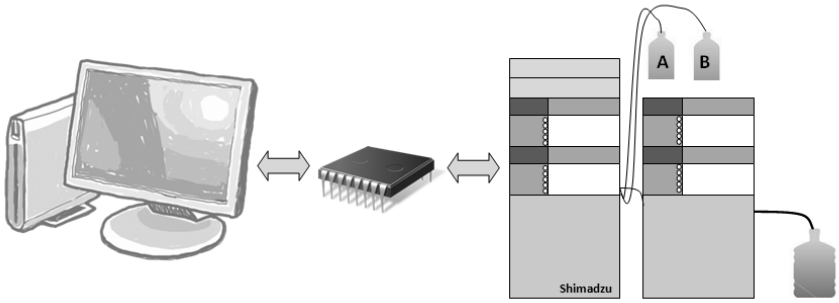
Appendix A-7 TFC method steps

- 0.5 ml of serially diluted extract or quercetin stock solution
- + 2ml distilled water
- (when t=0min.) + 0.15 ml 5% $NaNO_2$
- = Kept at dark for 5 min
- (when t=5 min.) + 0.15 ml 10% $AlCl_3$
- =Kept at dark for 6 min
- (when t=11 min.) + 1 ml 1M NaOH
- + 1.2 ml distilled water
- = Color change from blue to pink
- =Read at 510 nm relative to blank solution by spectrophotometer

Appendix A-8 Solvent flow rate for HPLC method

Time (Min)	Solvent	Solvent % flow rate
0.01	B	6
6	B	6
18	B	25
19	B	60
21	B	60
23	B	25
25	B	6
26	STOP	-

Appendix A-9 HPLC figure with solvents and the computer



Appendix B. Results of Analytical Methods

Appendix B-1 RSA values for MC1

Initial Concentration (mg/ml)	Final concentration(mg/ml)	AVERAGE RSA % MC1	STD Dev
0.000	0.000	0.000	0.000
5.997	0.400	13.990	2.174
11.994	0.800	25.214	1.907
17.992	1.199	35.436	1.550
35.983	2.399	63.454	4.610
53.975	3.598	83.694	1.156
71.967	4.798	90.116	1.469
108.347	7.223	91.646	1.671

Appendix B-2 RSA values for MC2

Initial Concentration (mg/ml)	Final concentration(mg/ml)	AVERAGE RSA % MC2	STD Dev
0.000	0.000	0.000	0.000
5.982	0.399	10.604	1.476
17.946	1.196	25.470	2.403
35.892	2.393	47.481	2.682
53.838	3.589	65.561	2.515
71.783	4.786	78.154	1.774
106.789	7.119	83.942	1.510
145.170	9.678	85.483	2.567

Appendix B-3 RSA values for MC3

Initial Concentration (mg/ml)	Final concentration(mg/ml)	AVERAGE RSA % MC3	STD Dev
0.000	0.000	0.000	0.000
3.047	0.203	9.138	0.575
9.142	0.609	29.918	1.351
18.283	1.219	52.297	1.535
36.567	2.438	85.201	1.560
54.850	3.657	94.954	1.118
73.133	4.876	96.269	1.345

Appendix B-4 RSA values for DC1

Initial Concentration (mg/ml)	Final concentration(mg/ml)	AVERAGE RSA % DC1	STD Dev
0.000	0.000	0.000	0.000
1.218	0.081	13.850	1.682
2.436	0.162	32.996	1.940
4.872	0.325	61.614	2.118
7.308	0.487	78.951	1.636
9.744	0.650	90.798	1.349
14.617	0.974	95.426	1.245
19.489	1.299	96.239	1.567

Appendix B-5 RSA values for DC2

Initial Concentration (mg/ml)	Final concentration(mg/ml)	AVERAGE RSA % DC2	STD Dev
0.000	0.000	0.000	0.000
1.574	0.105	13.477	0.692
3.149	0.210	27.359	3.439
6.297	0.420	48.334	3.289
12.594	0.840	81.159	2.036
18.892	1.259	92.379	1.864
25.189	1.679	94.719	1.987

Appendix B-6 RSA values for DC3

Initial Concentration (mg/ml)	Final concentration(mg/ml)	AVERAGE RSA % DC3	STD Dev
0.000	0.000	0.000	0.000
1.333	0.089	16.548	1.236
2.666	0.178	32.758	1.263
5.331	0.355	59.768	2.091
7.997	0.533	78.336	1.575
10.663	0.711	90.980	1.865
15.994	1.066	95.172	1.024

Appendix B-7 Trolox ABS values and final concentration for standard curve

Final µ M TE	ABS AVE	% inhibition	std dev
20	0.108	84%	0.01
10	0.400	41%	0.03
5	0.529	22%	0.02
4	0.559	18%	0.03
2	0.619	9%	0.03

Appendix B-8 Gallic acid ABS values and final concentration for standard curve

GALLIC ACID STD			
Initial conc. Mg/ml	Final Conc.mg/ml	ABS ave	stdev
0.000	0.000	0.000	0.000
0.050	0.002	0.109	0.003
0.100	0.005	0.246	0.008
0.200	0.009	0.528	0.012
0.300	0.014	0.763	0.053
0.400	0.018	0.996	0.026

Appendix B-9 Quercetin absorbance values and final concentration for standard curve

QUERCETIN			
Initial conc. mg/ml	Final conc. mg/ml	ABS Ave	Std Dev
0.000	0.000	0.000	0.000
0.100	0.010	0.152	0.021
0.200	0.020	0.346	0.016
0.300	0.030	0.491	0.011
0.400	0.040	0.703	0.006
0.500	0.050	0.861	0.012

Appendix B-10 Area values for (-)-epicatechin standard curve

Epicatechin (µg/ml)	Ave. Area
25	570239±3395
50	1163462±2548
75	1765618±6629
100	2369082±2860
125	2965666±21586

Appendix B-11 Area values for (+)-catechin standard curve

Catechin (µg/ml)	Ave. Area
6.25	72861±224
12.5	146665±1326
25	306096±2772
50	646120±247