İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

CHANGES IN ANTIOXIDANT CAPACITY OF CHOCOLATE ADDED WITH ALMONDS

M.Sc. Thesis by
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Department : Food Engineering

Programme : Food Engineering

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BADEM İLAVE EDİLEN ÇİKOLATALARDA ANTİOKSİDAN KAPASİTE DEĞİŞİMLERİNİN İNCELENMESİ

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FOREWORD

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ABBREVIATIONS

ABTS	**:** 2,2- azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt
ANOVA	**:** Analysis of Variance
CE	**:** Catechin equivalents
CUPRAC	**:** Cupric Reducing Antioxidant Capacity
DM	**:** Dry matter
DPPH	**:** 1,1-Diphenyl-2- picrylhydrazyl
ECE	**:** (-)-epicatechin equivalents
FRAP	**:** Ferric Reducing Antioxidant Power
GAE	**:** Gallic acid equivalents
QDA	**:** Quantitative Descriptive Analysis
TEAC	**:** Trolox Equivalent Antioxidant Capacity
TPTZ	**:** 2,4,6-tripyridyl-s-triazine

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CHANGES IN ANTIOXIDANT CAPACITY OF CHOCOLATE ADDED WITH ALMONDS

SUMMARY

The studies of cocoa and cocoa derived products have become an area of interest according to their health-promoting properties. Many epidemiological studies in recent years suggest that consumption of cocoa and cocoa derived products such as cocoa powder, cocoa liquor and dark chocolate can play an important role in preventing cancer and cardiovascular diseases. The health-promoting properties of cocoa were attributed to their phenolic compounds, mainly flavonoids. Flavonoids present in cocoa include mainly proanthocyanidins, flavonols and anthocyanins. High flavonoid content of cocoa is characterized with flavan-3-ols or flavonols including monomeric forms, (-)-epicatechin, (+)-catechin and the polymeric forms (procyanidins).

On the other hand, almond has a lower phenolic content and antioxidant capacity when compared to cocoa derivatives. Moreover, most of the almond phenolics are deposited in the skin of almonds. Besides, almonds are unusual according to their high protein and α-tocopherol contents compared to other nuts. They are often used in the chocolate and confectionary industries.

The nutritional value of cocoa and cocoa derivatives created a great interest in the effect of processing on their valuable compounds showing antioxidant capacity. The phenolic content of cocoa derived products is largely dependent upon the cocoa variety, cultivar, origin, agricultural and postharvest practices and processing. Therefore, understanding the effect of all the manufacturing processes from cocoa bean to chocolate on the total phenolic and flavonoid content and antioxidant capacity is crucial.

In this study, dark chocolate was produced by using three different formulations; 65% cocoa and 15% almond content (formulation A), 65% cocoa and 7.5% almond content (formulation B) and 65% cocoa content (control, formulation C) in a pilot scale laboratory plant. Deskinned almond paste was used in formulation A and B. In general, chocolate manufacturing include the five basic steps of mixing, refining, conching, tempering, and moulding and demoulding. In this study, three different dark chocolate samples were compared for their total phenolic contents, total flavonoid contents and total antioxidant capacities using different spectrophotometric methods such as 2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 1,1-diphenyl-2- picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and copper reducing antioxidant capacity (CUPRAC) in all steps of chocolate manufacturing.

Prior to spectrophotometric analysis, the best extraction solvent out of 70% methanol and 80% methanol was investigated by analyzing the amount of total phenolics, total flavonoids and antioxidant capacities with ABTS, DPPH, CUPRAC and FRAP methods of cocoa beans. Total phenolic contents of cocoa beans in 80% methanol extracts were 34% higher than that of 70% methanol extracts. Besides, total

flavonoid contents of cocoa beans in 80% methanol extracts were 30% higher than that of 70% methanol extracts. ABTS and DPPH results of cocoa bean samples showed that 80% methanol extracts yielded with 9% and 39% higher values than 70% methanol extracts, respectively. Similarly, CUPRAC and FRAP results showed that 80% methanol extracts were 11% and 30% higher compared to the 70% methanol extracts. As a result, 80% methanol was selected as the extraction solvent for spectrophotometric analysis.

The results indicated that highest total phenolic content was obtained in formulation C (726.95 mg CE/100 g dry matter (DM)), followed by formulation B (704.22 mg CE/100 g DM) and formulation A (485.84 mg CE/100 g DM) in mixing process. Similarly, highest total flavonoid content was observed in formulation C (573.81 mg CE/100 g DM), followed by formulation B (519.07 mg CE/100 g DM) and formulation A (437.85 mg CE/100 g DM). According to the results, the highest total antioxidant activity values were measured by CUPRAC method for each formulation. Formulation C had an average total antioxidant activity of 9836.63 µmol TEAC/100 g DM, whereas formulation B and A had activity values of 7852.28 µmol TEAC/100 g DM and 6798.04 µmol TEAC/100 g DM, respectively. The results of the ABTS method showed that formulation C had the highest average total antioxidant value of 6637.10 µmol TEAC/100 g DM, followed by 5774.70 µmol TEAC/100 g DM (formulation B) and 5275.22 µmol TEAC/100 g DM (formulation A). The lowest antioxidant activity values were obtained by DPPH method which indicated 4493.97 µmol TEAC/100 g DM as the highest value for formulation C, followed by formulation B (4153.78 µmol TEAC/100 g DM) and formulation A (3283.67 µmol TEAC/100 g DM). On the other hand, FRAP method also resulted in lower activity values in comparison with ABTS and CUPRAC methods (3614.12 µmol TEAC/100 g DM for Formulation A, 4361.84 µmol TEAC/100 g DM for Formulation B, and 4834,42 µmol TEAC/100 g DM for Formulation C). According to one way ANOVA variance analysis test, the differences among different chocolate formulations were statistically significant ($P<0.05$) in mixing process. The total phenolic content, total flavonoid content and antioxidant capacity values were observed in the order of formulation C, B and A.

In refining step of the chocolate production, it was observed that the total phenolic content, total flavonoid content and the total antioxidant capacity significantly increased compared to the mixing step according to the reduction of particle size. The results indicated that highest total phenolic content was observed in formulation C (801.56 mg CE/100 g DM, followed by formulation B (705.43 mg CE/100 g DM) and formulation A (622.48 mg CE/100 g DM) in refining process. Similarly, highest total flavonoid content was observed in formulation C (759.65 mg CE/100 g DM), followed by formulation B (594.26 mg CE/100 g DM) and formulation A (560.35 mg CE/100 g DM). According to the results, the highest total antioxidant activity values were measured by CUPRAC method for each formulation. Formulation C had an average total antioxidant activity of 10395.96 µmol TEAC/100 g DM, whereas formulation B and A had activity values of 9514.55 µmol TEAC/100 g DM and 8694.41 µmol TEAC/100 g DM , respectively. The results of the ABTS method showed that formulation C had the highest average total antioxidant value of 7817.07 µmol TEAC/100 g DM , followed by 7504.43 µmol TEAC/100 g DM (formulation B) and 6275.29 µmol TEAC/100 g DM (formulation A). The lowest antioxidant activity values were observed by DPPH method which indicated 4931.29 µmol TEAC/100 g DM as the highest value for formulation C, followed by formulation B

(4197.56 μmol TEAC/100 g DM) and formulation A (3765.78 μmol TEAC/100 g DM). On the other hand, FRAP method also resulted in lower activity values in comparison with ABTS and CUPRAC methods (4458.71 μmol TEAC/100 g DM for formulation A, 4597.15 μmol TEAC/100 g DM for formulation B, and 5077.56 μmol TEAC/100 g DM for formulation C). According to one way ANOVA variance analysis test, the differences among different chocolate formulations were statistically significant ($P<0.05$) in refining process. Similar to the mixing process, the total phenolic content, total flavonoid content and antioxidant capacity values were obtained in the order of formulation C, B and A in refining process.

In conching process, the trend of chocolate phenolic and flavonoid contents and total antioxidant capacity values were reduced based on heat treatment (70°C). Although particle size reduction increases *in vitro* antioxidant capacity in refining process, the thermal stability reduces. The decrease of the antioxidant capacity in the following conching and tempering steps might be resulted from degradation of some antioxidant compounds due to reduced thermal stability. The results indicated that highest total phenolic content was observed in formulation C (750.78 mg CE/100 g DM), followed by formulation B (617.42 mg CE/100 g DM) and formulation A (532.61 mg CE/100 g DM). Similarly, formulation C had highest total flavonoid content (567.28 mg CE/100 g DM) and formulation A and formulation B gave lower results of 30% and 14% compared to the control sample, respectively. According to the results, the highest total antioxidant activity values were obtained by CUPRAC method for each formulation. Formulation C had an average total antioxidant activity of 10022.03 μmol TEAC/100 g DM, whereas formulation B and A had an activity of 8612.71 μmol TEAC/100 g DM and 6741.48 μmol TEAC/100 g DM, respectively. The results of the ABTS method showed that formulation C had the highest average total antioxidant value of 6016.78 μmol TEAC/100 g DM and 24% and 11% lower antioxidant capacities were observed for formulation A and formulation B compared to the control sample, respectively. The lowest antioxidant activity values were obtained by DPPH method which indicated 4331.42 μmol TEAC/100 g DM as the highest value for formulation C, followed by formulation B (3675.19 μmol TEAC/100 g DM) and formulation A (2801.56 μmol TEAC/100 g DM). On the other hand, FRAP method also resulted with lower activity values in comparison with ABTS and CUPRAC methods. Similarly to the DPPH and CUPRAC methods, antioxidant capacities of formulation A and B were about 32% and 16% lower than the control sample (4929.54 μmol TEAC/100 g DM), respectively. According to one way ANOVA variance analysis test, the differences among different chocolate formulations were statistically significant ($P<0.05$) in conching process and phenolic compounds were observed in the order of formulation C, B and A.

In tempering process, there were significant reductions in the total phenolic and flavonoid contents and antioxidant capacities compared to conching step according to continuous heating (46°C) and cooling (32°C) of the chocolate products, but the trend of the chocolate formulations were the same. The total phenolic contents of formulation A and formulation B were found to be about 31% and 11% lower than the control sample (610.31 mg CE/100 g DM), respectively. Similarly, the results indicated that highest total flavonoid content was obtained in formulation C (425.91 mg CE/100 g DM), followed by formulation B (402.60 mg CE/100 g DM) and formulation A (338.84 mg CE/100 g DM). The highest total antioxidant capacity values were obtained by CUPRAC method for each formulation (6741.48 μmol TEAC/100 g DM for formulation C, 6573.36 μmol TEAC/100 g DM for formulation

B and 5428.00 μmol TEAC/100 g DM for formulation A in descending order). The results of the ABTS method indicated that about 26% and 12% lower values for antioxidant capacities of formulation A and formulation B were obtained as a comparison with the control sample (5244.07 μmol TEAC/100 g DM), respectively. The lowest antioxidant activity values were obtained by DPPH method which indicated 3686.26 μmol TEAC/100 g DM as the highest value for formulation C, followed by formulation B (3322.42 μmol TEAC/100 g DM) and formulation A (2506.66μmol TEAC/100 g DM). Besides, FRAP method also resulted with lower activity values in comparison with ABTS and CUPRAC methods. Similar to results obtained by ABTS and DPPH methods, in tempering process the antioxidant capacities of formulation A and formulation B were 27% and 14% lower than the control sample (4037.50 μmol TEAC/100 g DM), respectively. According to one way ANOVA variance analysis test, the differences among different chocolate formulations were statistically significant ($P<0.05$) in tempering process. The total phenolic content, total flavonoid content and antioxidant capacity values were obtained in the order of formulation C, B and A in tempering process.

The data of the chocolate final products were not very clear due to the difficulties of lipid removal from the samples using the same method for comparison. Only the CUPRAC method indicated the same trend with the tempering process. According to the results, the highest total antioxidant activity values were obtained by CUPRAC method for each formulation. Formulation A had an average total antioxidant activity of 6540.72 μmol TEAC/100 g DM, whereas formulation B and C had activity values of 7076.72 μmol TEAC/100 g DM and 6668.15 μmol TEAC/100 g DM, respectively.

In the study, the total phenolic and flavonoid contents and antioxidant capacities of the major ingredients used in each production were also measured to evaluate if there were any differences resulting from the ingredients used in each formulation between the two productions. Paired t-test analysis was performed between the first and the second production major ingredients to compare the effect of using different ingredients on total phenolic content, total flavonoid content and total antioxidant activity. The results of t-test indicated that the differences between the first and the second production were found to be statistically significant ($P<0.05$).

In the sensory analysis section, The Quantitative Descriptive Sensory Analysis Technique was used to compare all the sensory characteristics of chocolate samples. Twelve trained panelists from Istanbul Technical University, Food Engineering Department participated to the sensory panels. Panelists identified and defined the perceived sensory characteristics, and furthermore they reached to consensus on terminology for each sensory property. Panelists were then trained to evaluate the intensity of each characteristic using reference chocolate products on a 0-7 point category scale for 6 h. The panel agreed upon a total of 20 different descriptive terms in chocolate samples.

The Kruskal-Wallis nonparametric test was used to analyze the sensory analysis data of two sensory panels to determine whether there were significant differences ($P<0.05$) between different chocolate samples for predetermined descriptives tested by the panelists.

The results of this study indicated that there were statistically significant differences between three formulations of chocolate for the characteristics of almond odor ($H(2) = 24.331$, $p = 0.000$), almond aroma ($H(2) = 14.851$, $p = 0.001$), bitter taste ($H(2) = 6.077$, $p = 0.048$), oily taste $H(2) = 18.213$, $p = 0.000$), melting rate ($H(2) = 7.267$, $p = 0.026$), firmness ($H(2) = 8.514$, $p = 0.014$), and oily mouthfeel ($H(2) = 13.455$, $p = 0.001$) depending on the almond content.

BADEM İLAVE EDİLEN ÇİKOLATALARDA ANTİOKSİDAN KAPASİTE DEĞİŞİMLERİNİN İNCELENMESİ

ÖZET

Kakao ve kakao ürünlerine ait çalışmalar, bu ürünlerin sağlığı geliştirici özelliklerinden dolayı ilgi çekmektedir. Son yıllarda yapılan epidemiyolojik çalışmalar kakao ve kakao tozu, kakao likörü ve bitter çikolata gibi kakao ürünlerinin tüketilmesinin kanseri, kalp ve damar hastalıklarını önlemede önemli rolünün olduğunu göstermektedir. Kakaonun sağlığı geliştirici özellikleri fenolik bileşiklerine, temel olarak flavonoidlere dayandırılmaktadır. Kakaoda bulunan flavonoidleri daha çok proantosiyanidinler, flavonoller ve antosiyaninler oluşturmaktadır. Kakaonun yüksek flavonoid içeriği monomerik formdaki (-)-epikateşin, (+)-kateşin ve polimerik formdaki prosiyanidinleri içeren flavan-3-oller veya flavonoller ile tanımlanmaktadır.

Buna karşılık, bademin fenolik içeriği ve antioksidan kapasitesi kakao türevlerine göre daha düşüktür. Ayrıca badem fenoliklerinin büyük bir kısmı bademin kabuğunda depolanmıştır. Bunun yanında, badem diğer kuruyemişlerle karşılaştırıldığında yüksek protein ve α-tokoferol içeriğiyle farklılık göstermektedir. Çikolata ve şekerleme endüstrilerinde badem sıkça kullanılmaktadır.

Kakao ve kakao ürünlerinin besin değeri prosesin antioksidan kapasite gösteren değerli bileşiklerin üzerine etkisi konusunda ilgi çekmektedir. Kakao türevi ürünlerinin fenolik içeriği kakaonun cinsine, çeşidine, kökenine, tarımsal ve hasat sonrası uygulamalara ve prosese büyük ölçüde bağlıdır. Bu nedenle, kakao çekirdeğinden çikolataya kadar bütün üretim basamaklarının toplam fenolik ve flavonoid içerik ile antioksidan kapasite üzerine etkisinin anlaşılması çok önemlidir.

Bu çalışmada, bitter çikolata üç farklı formülasyon olarak; %65 kakao ve %15 badem içeriği (formülasyon A), %65 kakao ve %7,5 badem içeriği (formülasyon B), %65 kakao içeriği (kontrol, formülasyon C) pilot ölçekli laboratuar ortamında üretilmiştir. Formülasyon A ve B kabuğu soyulmuş badem içermektedir. Genel olarak çikolata üretimi, karıştırma, inceltme, konçlama, temperleme, kalıplama ve kalıptan dökme aşamalarından oluşmaktadır. Bu çalışmada, üç farklı bitter çikolata numunesinin toplam fenolik içerikleri, toplam flavonoid içerikleri ve ABTS, DPPH, CUPRAC ve FRAP gibi farklı spektrofotometrik metodlar kullanılarak toplam antioksidan kapasiteleri çikolata üretiminin her aşamasında karşılaştırılmıştır.

Spektrofotometrik analizlerin öncesinde, %70 metanol ve %80 metanol kullanılarak en iyi ekstraksiyon çözgeninin bulunabilmesi için toplam fenolik miktarı, toplam flavonoid miktarı ve ABTS, DPPH, CUPRAC ve FRAP metodları kullanılarak antioksidan kapasitesi kakao çekirdeklerinde analizlenmiştir. Kakao çekirdekleri için %80 metanol ekstraktındaki toplam fenolik içerikleri %70 metanol ekstraktlarına oranla %34 daha fazladır. Bunun yanında, kakao çekirdeklerinin flavonoid içeriği %80 metanol ekstraklarında %70 metanol ekstraklarına göre %30 daha fazladır. Kakao çekirdeği numunelerinin ABTS ve DPPH sonuçları %80 metanol

ekstraklarının %70 metanol ekstraklarına göre sırasıyla %9 ve %39 daha yüksek değerler verdiğini göstermektedir. Benzer olarak, CUPRAC ve FRAP sonuçları da %80 metanol ekstraklarının %70 metanol ekstraklarına göre sırasıyla %11 ve %30 daha yüksek değerler verdiğini göstermektedir. Sonuç olarak, spektrofotometrik analizler için ekstraksiyon çözgeni olarak %80 metanol seçilmiştir.

Sonuçlar karıştırma aşamasında en yüksek toplam fenolik içeriğin formülasyon C'den (726,95 mg CE/100 g kuru madde (KM)) elde edildiğini ve bunu formülasyon B (704,22 mg CE/100 g KM) ve formülasyon A'nın (485,84 mg CE/100 g KM) takip ettiğini belirtmektedir. Aynı şekilde, en yüksek toplam flavonoid içeriğin de formülasyon C'den (573,81 mg CE/100 g KM) elde edildiğini ve bunu formülasyon B (519,42 mg CE/100 g KM) ve formülasyon A'nın (437,85 mg CE/100 g KM) izlediği belirtilmiştir. Sonuçlara göre, her bir formülasyon için en yüksek toplam antioksidan aktivite değerleri CUPRAC metoduyla elde edilmiştir. Formülasyon C 9836,63 µmol TEAC/100 g KM ortalama toplam antioksidan aktivitesine sahipken, formülasyon B ve A sırasıyla 7852,28 µmol TEAC/100 g KM ve 6798,04 µmol TEAC/100 g KM aktiviteye sahiptir. ABTS metodunun sonuçları formülasyon C'nin 6637,10 µmol TEAC/100 g KM ile en yüksek ortalama toplam antioksidan değerine sahip olduğunu ve bu değeri 5774,70 µmol TEAC/100 g KM (formülasyon B) ve 5275,22 µmol TEAC/100 g KM (formülasyon A) takip ettiğini göstermektedir. En düşük antioksidan aktivite değerleri DPPH metodu ile bulunmuştur. En yüksek değer 4493,97 µmol TEAC/100 g KM ile formülasyon C için bulunmuş ve bunu formülasyon B (4153,78 µmol TEAC/100 g KM) ve formülasyon A (3283,67 µmol TEAC/100 g KM) değerleri izlemiştir. Diğer taraftan, FRAP metodu da ABTS ve CUPRAC metodlarıyla karşılaştırıldığında (formülasyon A için 3614,12 µmol TEAC/100 g KM, formülasyon B için 4361,84 µmol TEAC/100 g KM ve formülasyon C için 4834,42 µmol TEAC/100 g KM) düşük antioksidan aktivite değerleri vermektedir. ANOVA varyans analizi testine göre, karıştırma aşamasında çikolata formülasyonları arasındaki farklılıklar istatistiksel olarak önemli ($P<0,05$) sonuç vermektedir. Toplam fenolik içerik, toplam flavonoid içerik ve antioksidan aktivite değerleri azalan sırayla formülasyon C,B ve A'da bulunmuştur.

Çikolata üretiminin inceltme aşamasında, partikül boyutunun küçülmesine bağlı olarak toplam fenolik içeriğin, toplam flavonoid içeriğin ve toplam antioksidan kapasitenin karıştırma aşamasıyla karşılaştırıldığında önemli ölçüde yükseldiği bulunmuştur. Sonuçlar toplam fenolik içeriği formülasyon C'de (801,56 mg CE/100 g KM) en yüksek değeri verdiğini ve bunu formülasyon B (705,43 mg CE/100 g KM) ve formülasyon A'nın (622,48 mg CE/100 g KM) takip ettiğini göstermektedir. Benzer şekilde, en yüksek flavonoid içerik formülasyon C'de (759,65 mg CE/100 g KM) bulunmakta ve bunu formülasyon B (594,26 mg CE/100 g KM) ve formülasyon A (560,35 mg CE/100 g KM) takip etmektedir. Sonuçlara göre, her formülasyonda CUPRAC metodu en yüksek antioksidan aktivite değerlerini vermektedir. Formülasyon C 10395,96 µmol TEAC/100 g KM ortalama antioksidan aktivite değerine sahipken formülasyon B ve A sırasıyla 9514,55 µmol TEAC/100 g ve 8694,41 µmol TEAC/100 g değerlerine sahiptir. ABTS metodunun sonuçları da formülasyon C'nin en yüksek ortalama antioksidan aktivitesi (7817,07 µmol TEAC/100 g) değerini verdiğini, bunu formülasyon B (7504,43 µmol TEAC / 100 g) ve formülasyon A'nın (6275,29 µmol TEAC/100 g) izlediğini göstermektedir. En düşük antioksidan aktivitesi değerleri DPPH metoduyla elde edilmiştir. En yüksek değer 4931,29 µmol TEAC/100 g KM ile formülasyon C için bulunmuş ve bunu formülasyon B (4197,56 µmol TEAC/100 g KM) ve formülasyon A (3765,78 µmol

TEAC/100 g KM) izlemiştir. Bunun yanında, FRAP metodu da ABTS ve CUPRAC metodları ile karşılaştırıldığında daha düşük aktivite değerleri vermiştir (Formülasyon A için 4458,71 µmol TEAC/100 g KM, formülasyon B için 4597,15 µmol TEAC/100 g KM, formülasyon C için 5077,56 µmol TEAC/100 g KM). ANOVA varyans analizi testine göre, inceltme aşamasında çikolata formülasyonları arasındaki farklılıklar istatistiksel olarak önemli ($P<0,05$) sonuç vermektedir. Karıştırma prosesine benzer şekilde, toplam fenolik içerik, toplam flavonoid içerik ve antioksidan kapasite en yüksekten düşüğe sırasıyla formülasyon C, B ve A'da gözlenmiştir.

Konçlama aşamasında, çikolatanın fenolik ve flavonoid içeriği ve antioksidan kapasitesi sıcaklık uygulamasına bağlı olarak (70°C) düşme eğilimindedir. İnceltme aşamasında partikül boyutunun düşmesi *in vitro* antioksidan kapasiteyi arttırmasına rağmen, ısıl stabiliteyi düşürmektedir. Antioksidan kapasitenin devam eden konçlama ve temperleme aşamalarında düşmesi bazı antioksidan bileşiklerin ısıl stabilitesinin azalması sonucunda degradasyonu ile sonuçlanabilmektedir. Sonuçlar en yüksek toplam fenolik içeriğin formülasyon C'de (750,78 mg CE/100 g KM) elde edildiğini ve bunu formülasyon B (617,42 mg CE/100 g KM) ve formulasyon A'nın (532,61 mg CE/100 g KM) takip ettiğini göstermektedir. Benzer şekilde, formülasyon C en yüksek toplam flavonoid içeriğine (567,28 mg CE/100 g KM) sahiptir ve formülasyon A ve B kontrol örneği ile karşılaştırıldığında sırasıyla %30 ve %14 daha düşük sonuçlar vermektedir. Sonuçlara göre CUPRAC metoduyla her formülasyon için en yüksek toplam antioksidan aktivite sonuçlarına ulaşılmaktadır. Formülasyon C 10022,03 µmol TEAC/100 g KM ortalama toplam antioksidan aktivite değerine sahipken formülasyon B ve A sırasıyla 8612,71 µmol TEAC / 100 g KM ve 6741,48 µmol TEAC/100 g KM aktiviteye sahiptir. ABTS metoduna göre formülasyon C en yüksek toplam antioksidan değerine (6016,77 µmol TEAC/100 g KM) sahipken formülasyon A ve B'nin antioksidan kapasiteleri sırasıyla kontrol örneğiyle karşılaştırıldığında %24 ve %11 daha düşük değerler vermektedir. DPPH metodu en düşük antioksidan aktiviteyi veren metot olarak en yüksek değeri formülasyon C için (4331,42 µmol TEAC/100 g KM) vermekte ve bunu formülasyon B (3675,19 µmol TEAC/100 g KM) ve formülasyon A (2801,56 µmol TEAC/100 g KM) izlemektedir. Diğer yandan, FRAP metodu da ABTS ve CUPRAC metodları ile karşılaştırıldığında daha düşük aktivite sonuçları vermektedir. DPPH ve CUPRAC metodlarına benzer şekilde, formülasyon A ve B'nin antioksidan kapasiteleri kontrol örneğine göre (4929,54 µmol TEAC/100 g KM) sırasıyla yaklaşık %32 ve %16 daha düşüktür. ANOVA varyans analizi testine göre, konçlama prosesinde çikolata formülasyonları arasındaki farklılıklar istatistiksel olarak önemlidir ($P<0,05$) ve fenolik bileşikler azalan sırasıyla formülasyon C,B ve A'da bulunmuştur.

Temperleme aşamasında, toplam fenolik ve flavonoid içeriğinde ve antioksidan kapasitesinde konçlama aşamasıyla karşılaştırıldığında ürünün sürekli ısıtılması (46°) ve soğutulmasına (32°C) bağlı olarak önemli bir düşüş olmakta, fakat çikolata formülasyonlarının eğilimi değişmemektedir. Formülasyon A ve B'nin toplam fenolik içeriklerinin kontrol örneğine göre (610,31 mg CE/100 g KM) sırasıyla yaklaşık %31 ve %11 daha düşük olduğu bulunmuştur. Benzer şekilde, sonuçlar en yüksek toplam flavonoid içeriğinin formülasyon C'de (425,91 mg CE/100 g KM) bulunduğunu ve bunu formülasyon B (402,60 mg CE/100 g KM) ve formülasyon A'nın (338,84 mg CE/100 g KM) izlediğini belirtmektedir. En yüksek toplam antioksidan kapasite değerleri CUPRAC metoduyla bulunmuştur (Formülasyon C için 6741,48 µmol TEAC/100 g KM, formülasyon B ve A için de azalan sırayla

6573,36 µmol TEAC/100 g KM ve 5428,00 µmol TEAC/100 g KM). ABTS metodu sonuçları da formülasyon A ve B için kontrol örneği ile karşılaştırıldığında (5244,07 µmol TEAC/100 g KM) sırasıyla yaklaşık %26 ve %12 daha düşük değerler vermektedir. En düşük antioksidan aktivite değerleri DPPH metodunda elde edilmiştir ve bu metodla en yüksek değeri 3686,26 µmol TEAC/100 g KM ile formülasyon C vermiştir. Bunu formülasyon B (3322,42 µmol TEAC/100 g KM) ve formülasyon A (2506,66 µmol TEAC/100 g KM) izlemektedir. Bunun yanında, FRAP metodu da ayrıca ABTS ve CUPRAC metodlarına göre daha düşük aktivite değerleri ile vermektedir. ABTS ve DPPH metodlarına benzer olarak, formülasyon A ve formülasyon B temperleme prosesinde kontrol örneği (4037,50 µmol TEAC/100 g KM) ile karşılaştırıldığında sırasıyla yaklaşık %27 ve %14 daha düşük sonuçlar vermiştir. ANOVA varyans analizi testine göre, çikolata formülasyonları arasındaki farklılıklar istatistiksel olarak önemlidir ($P<0,05$). En yüksek toplam fenolik içerik, toplam flavonoid içerik ve antioksidan kapasite değerleri sırasıyla C,B ve A formülasyonlarında elde edilmiştir.

Çikolata son ürünlerdeki veriler, karşılaştırılabilirlik açısından aynı metod kullanılarak yağın örneklerden uzaklaştırılmasında yaşanan zorluklardan dolayı çok açıklayıcı değildir. Sadece CUPRAC metodunun sonuçları temperleme prosesi ile aynı eğilimi göstermektedir. Sonuçlara göre, en yüksek antioksidan aktivite değerleri her formülasyon için yine CUPRAC metoduyla elde edilmiştir. Formülasyon A 6540,72 µmol TEAC/100 g KM ortalama toplam antioksidan aktiviteye sahipken, formülasyon B ve C sırasıyla 7076,72 µmol TEAC/100 g KM ve 6668,15 µmol TEAC/100 g KM aktivitelerine sahiptir.

Bu çalışmada ayrıca çikolata üretiminde kullanılan temel malzemelerin toplam fenolik ve flavonoid içerikleri ve antioksidan kapasiteleri, tekrarlanan iki üretimdeki her formülasyonda kullanılan malzemelerden kaynaklanan bir farklılık olup olmadığını değerlendirebilmek için ölçülmüştür. Birinci ve ikinci üretimde kullanılan farklı malzemelerin toplam fenolik içerik, toplam flavonoid içerik ve toplam antioksidan aktivite üzerine etkisinin değerlendirilebilmesi için birinci ve ikinci üretimde kullanılan hammaddeler arasında T testi analizi gerçekleştirilmiştir. T testi analizi sonucunda birinci ve ikinci üretimde kullanılan hammaddeler arasında istatistiksel olarak önemli farklılık bulunmuştur ($P<0,05$).

Duyusal analiz bölümünde, çikolata numunelerinin duyusal özelliklerini karşılaştırabilmek için Kantitatif Tanımlama Analizi tekniği kullanılmıştır. İstanbul Teknik Üniversitesi Gıda Mühendisliği Bölümü'nden oniki eğitimli panelist duyusal panele katılmıştır. Panelistler algılanan duyusal özellikleri teşhis etmiş ve tanımlamış, sonrasında da her duyusal özellik için terminoloji üzerine fikir birliği oluşturulmuştur. Daha sonra, panelistler her özelliğin yoğunluğunu referans çikolata örnekleri kullanılarak 0-7 kategori skalasında değerlendirmeleri için 6 saat boyunca çalışmıştır. Panel çikolata örnekleri için toplam 20 farklı tanımlayıcı terim üzerinde anlaşmıştır.

Kruskal-Wallis nonparametrik testi, panelistler tarafından önceden belirlenmiş tanımlayıcı özellikler için farklı çikolata örnekleri arasında anlamlı bir farklılık olup olmadığının belirlenmesi amacıyla iki duyusal panelin de verilerini analiz etmek için kullanılmıştır.

Bu çalışmanın sonucunda üç çikolata formülasyonu arasında badem içeriğine bağlı olarak, badem kokusu ($H(2) = 24{,}331$, $p = 0{,}000$), badem aroması ($H(2) = 14{,}851$, $p = 0{,}001$), bitter tat ($H(2) = 6{,}077$, $p = 0{,}048$), yağlılık $H(2) = 18{,}213$, $p = 0{,}000$), erime hızı ($H(2) = 7{,}267$, $p = 0{,}026$), sertlik ($H(2) = 8{,}514$, $p = 0{,}014$) ve ağızda yağlılık hissi ($H(2) = 13{,}455$, $p = 0{,}001$) özelliklerinde istatistiksel olarak önemli farklılıklar ($P<0{,}05$) olduğu belirtilmiştir.

1. INTRODUCTION

Reactive oxygen species are involved in several human diseases like cancer (Azad et al., 2008; Federico et al., 2007), asthma (Henricks and Nijkamp, 2001), atherosclerosis (Bennett, 2001), inflammatory bowel disease (Rezaie et al., 2007) and Alzheimer's disease (Guidi et al., 2007). Antioxidants prevent or heal these diseases that are caused by reactive oxygen species (Uttara et al., 2009). There is a growing evidence that some of the foods can lead to reduction of oxidative damage in biological systems by their antioxidant potential.

Cocoa derived products named as cocoa powders, chocolate and other related products are polyphenol-rich foods derived from the seeds of *Theobroma cacao* L. (Sterculiaceae) that are widely consumed in all over the world. Chocolate contributes to 20% of the flavonoid intake by adults and this value is even higher for children in Germany. Chocolate is considered as the third source of antioxidants taken by the diet (100-107 mg/day) after the fruits (255 mg/day) and vegetables (233 mg/day) in diet (Vinson et al., 2006).

The studies of cocoa and cocoa derived products have become an interest for many years due to their health promoting properties. Cocoa polyphenols have been reported in many studies as bioactive compounds that carry out antioxidant, antiradical and anticarcinogenic properties (Schinella et al., 2010; Abbe Maleyki and Amin, 2008; Ren et al., 2003). The consumption of flavanol-rich cocoa products has been reported to improve endothelial function (Wang-Polagruto et al., 2006), reduce pro-inflammatory mediators (Sies et al., 2005), decrease platelet function (Murphy et al. 2003), increase dermal blood flow (Neukam et al., 2007), inhibit the proliferation of human breast cancer cells (Ramljak et al., 2005) and exert hypoglycemic properties (Tomaru et al., 2007).

Cocoa beans are rich source of polyphenols that their about 10% of the dry weight contribute to polyphenols and its derivative dark chocolate is considered as one of the major contributors of antioxidants (Rusconi and Conti, 2010). Polyphenols of cocoa and cocoa derived products can be classified into three main groups as proanthocyanidins (58%), flavan-3-ols (37%) and anthocyanins (4%) (Wollgast and

Anklam, 2000). Quantitatively the main polphenolic compound is (-)-epicatechin with 35% content in cocoa beans (Shahidi and Nazck, 2004). (+)-Catechin, traces of (+)-gallocatechin, (-)-epigallocatechin, (-)-epicatechin-3-gallate and numerous procyanidins (Adamson et al., 1999; Hammerstone et al., 2000), as well as small quantities of quercetin, quercetin glycosides, naringenin, luteolin, apigenin, clovamide and phenolic acids such as caffeic, ferulic, gallic and p-coumaric acid have also been found in cocoa products (Borchers et al., 2000; Sanchez-Rabaneda et al., 2003). Their affinity to form complexes with proteins enhances the complexity of cocoa products (Komes et al., 2009).

Almonds specifically known as *Prunus dulcis* are eaten raw, roasted and fried; but can also be used as ingredients in different products such as chocolate. They are also processed to make nutritional products such as almond milk used as a substitude cow's milk (Bartolemé et al., 2010). Compared to other nuts almonds are unusual for their high protein and α-tocopherol contents (Mandalari et al., 2008). According to USDA Nutrient database, almonds have 21.22% of protein content (USDA, 2010). In recent studies, it is suggested that polyphenols bind to proteins either covalently or non-covalently and reduce the total antioxidant capacity (Serafini et al., 2009; Ryan and Petit, 2010; Sharma et al., 2008; Komes et al., 2009).

Almonds are often used in chocolate and confectionary products as they contain phenolic compounds like dark chocolate. Although almond skin represents the 4% of the total almond weight it contains 70-100% of total phenols present in the nut (Milbury et al., 2006).

Chocolate processing has five main steps as mixing, refining, conching, tempering and moulding and demoulding steps (Afoakwa, 2010b). These process steps may have an effect on the total phenolic content, total flavonoid content and total antioxidant capacity. In the current literature there aren't any available data that give the effect of processing on dark chocolate.

The present study has three objectives; the first objective of this study was to investigate the effect of almond content of the dark chocolate total phenolic content, total flavonoid content and total antioxidant capacity. In order to investigate the almond effect three formulations of dark chocolate were prepared as dark chocolate with 65% cocoa and 15% almond content, dark chocolate with 65% cocoa and 7.5% almond content and dark chocolate with 65% cocoa content as a control sample for

comparison. The second objective was to investigate the effect of dark chocolate manufacturing steps on total phenolic content, total flavonoid content and total antioxidant capacity. The third objective was to analyze the sensory attributes for elucidating major sensorial differences between three chocolate formulations using the Quantitative Descriptive Analysis method.

This research thesis is presented as literature, materials and methods, results and discussion and conclusion parts. In the literature chapter, history of cocoa and chocolate, cocoa processing and technology, chocolate processing and technology and phenolic compounds of cocoa and chocolate were reviewed. Materials and methods section included detailed information about the chocolate processing performed at pilot scale laboratory plant. The detailed protocols followed to measure total phenolic content, total flavonoid content and total antioxidant capacity using ABTS, DPPH, CUPRAC and FRAP methods were also presented in materials and methods. Results and discussion part was divided into four main headlines including: solvent choice for spectrophotometric methods, phenolic contents of major chocolate ingredients, the effect of processing and almond content on chocolate phenolics and sensory analysis of products. In the conclusion part, a need for further investigation on the changes of healthy compounds was emphasized.

2. LITERATURE REVIEW

2.1. History of cocoa and chocolate

The term ‘cocoa’ comes directly from the word ‘cacao’ of the Aztec and Mayan languages. Chocolate is produced by using the cocoa beans, which are placed at the central part of the cocoa tree *Theobroma cacao*, belonging to South America and originated from the Amazon and Orinoco valleys. *Theobroma* meaning ‘food of the Gods’ contains four types: *Criollo*, 5% of world cocoa production; *Forastero*, more common, have smaller, flatter and purple beans; *Nacional*, grown in Ecuador, have fine flavor; hybrid of *Forastero* and *Trinitario*, more resistant to disease and have fine flavor (Fowler, 2009).

Thebroma cocoa has varieties in forest areas of South America which are grown between Cancer and Capricorn tropics. Forastero is the basic cocoa grows in Brasil and West Africa, whereas flavor cocoas are cultivated in Central and South America mostly as hybrids. Aztecs cultivated cocoa from South America through Caribbean islands in Mexico. Hernandos Cortez entered cocoa to Spain in the form of beverage and to Spanish Guinea as a crop. The Spanish took cocoa to Europe, but also affected the future economies of many West African countries by introducing the crops into Fernando Po in the seventeenth century. At present, West Africa produces 70% of world cocoa (Awua, 2002; Amoye, 2006; International Cocoa Organisation, ICCO, 2008).

Aztecs and Incas used cocoa beans as a currency for trade and they also produced a chocolate drink called *chocolatl*. *Chocolatl* was produced by roasting and grinding cocoa nibs, crashing with water and addition of ingredients like vanilla, spices and honey. Thus, the use of cocoa beans dates back at least 1400 years (Rössner, 1997). Although Columbus is known to have been discovered chocolate on his fourth journey in 1502 by locals of the island of Guanaja near Honduras, it was Hernando Cortez who really discovered the ancient fruit 17 years later in Mexico. He discovered the first plantations and recipe for the original chocolate drink. It was a

red, bitter and spicy drink called Xocoatl. It was consumed in the ceremonies of Aztecs. After Hernando Cortez returned to Spain with the first beans in 1528, its powerful medicinal properties and nutritional value soon realized (Dhoedt, 2008).

Chocolate drink underwent its first change preparing the foundations for the chocolate recipes of today in 1590. Spanish monks added honey, vanilla and sugar cane for the adaptation of the chocolate drink to European tastes. Later, chocolate was introduced by merchant traders and explorers in Italy, France, Prussia, the Low Countries and Switzerland. The first company that produced chocolate was developed in France by David Chaillou who made chocolate biscuits and cakes for the larger society (Dhoedt, 2008). Chocolate was faced with great skepticism by the Church in the early 17th century. There was no permission for the chocolate as it thought to cause a sinful and decadent diversion on clergy. Then the Church was forced to permit the consumption of chocolate for the wealthy that even drink their chocolate drink during the mass to sit out the lengthy service in 1660 (Dhoedt, 2008).

The chocolate plants were soon established by the Italians, Dutch and Portuguese and the Spanish monopoly became unholdable about the chocolate production, as the consumption of the chocolate became widespread during the 18th century. At this time the chocolate was still consumed in the liquid form or pressed blocks to be dissolved in water or milk for the foamy chocolate drink. The British Fry family built the first chocolate factory using hydraulic equipment for grinding the cocoa beans in 1728. Dr. James Baker built the first factory in US and in 1778 Frenchman Doret built the first automated machine for grinding cocoa beans. In 1828 the production of cocoa and chocolate was revolutionized by the Coenraad Van Houten that he separated the cocoa solids from cocoa butter. Defatted cocoa powder was easily soluble in water and in 1848 the first ‘eating chocolate’ was produced by the addition of cocoa butter and sugar to cocoa liquor (Dhoedt, 2008).

Plain eating chocolate bar was produced by the introduction of cocoa butter as an ingredient by Joseph Fry in the UK in 1847 (Beckett, 2000). As the chocolate processing became mechanized with development of cocoa presses for the production of cocoa powder and cocoa butter by Van Houten in 1828, the demand for cocoa sharply increased. Daniel Peter who had the idea of adding the milk powder to chocolate invented the first milk chocolate in 1875 in Switzerland. Another Swissman, Rudolphe Lindt invented the conching machine by the late 19th

century and produced a good quality chocolate with a smooth texture. In 1900 the price of the two important ingredients of chocolate decreased and the chocolate became accessible to the middle class (Dhoedt, 2008).

Chocolate production had spread from Central and South America to Africa and Asia. Smaller plantations and independent farms took place of large plants. Chocolate prices were reduced by the widespread production of chocolate all over the Europe and U.S. Countries like Belgium with fast manufacturing technologies and innovative marketing techniques. The big chocolate brands appeared at this time. Callebaut and Cacao Barry accepted as a standard for chocolate production for their high quality products. The world's most famous chocolate companies like Neuhaus and Godiva in Belgium, La Maison du Chocolat and Fauchon in France, Lindth, Suchard and Sprüng in Switzerland appeared in the 20th century. Jean Neuhaus found filling chocolate shells with cream and nut pastes in 1912 (Dhoedt, 2008).

As the prices of the chocolate ingredients decreased and the chocolate making process became more efficient by the 1930s and 1940s, chocolate had finally become available for larger population. It became the world's most popular small-sized snack (Afoakwa, 2010a). Annual consumption of chocolate confectionary in many European countries is about 8.0 kg/person (Webber, 2009; ICCO, 2008).

2.2. Cocoa Processing and Technology

2.2.1. Cocoa bean selection and quality criteria

There are some guidelines and quality criteria that chocolate manufacturers should follow to maintain consumer's loyalty to their products. The quality of the cocoa beans can be measured using two techniques. The first technique contains the following indicators for the quality evaluation: Degree of fermentatiton, moisture content (maximum 6%), number of defects, bean count (number of the beans/ 100 g), degree of mouldiness, flavor profile, color, fat content (minimum 52%), fat quality relating to percentage of free fatty acids (as oleic acids), shell content (10-12%), uniformity of bean size, insect and rodent infestation (Afoakwa, 2010b).

The second evaluation technique is based on the size of the beans using either the bean count (number of beans per 100 g) or the weight in grams of 100 beans. The cocoa prizes change according to their size in the international cocoa market. Cocoa

beans with smaller sizes contain lower amount of nibs, higher shell content, lower fat content and cheaper that cocoa beans with bigger sizes. Asian cocoa nibs contain higher shell content than West Africa cocoa nibs (Afoakwa, 2010b).

Degree of fermentation and defects can be measured by bean cut test. In bean cut test, 300 beans are randomly selected and cutted longitudinally. Cutted surfaces are analyzed for: Flat and shrunken beans, moldy beans, slaty beans, germinated beans, degree of insect and rodent infestination (Afoakwa, 2010b).

Flavor and taste of the final products are affected by all these factors. Good quality cocoa beans should be well fermented, dry and free from insects, contaminations/adulterations and rodent infestation. It should have a proper color (Afoakwa, 2010b).

2.2.2. Cleaning, breaking and winnowing

Cocoa beans are passed through the cleaning, breaking and winnowing process to have constant quality before processing. They should be cleaned from dirt and infestation, broken and deshelled properly. They should have constant size to obtain constant quality. Firstly, beans are sieved and all the extraneous materials like stones, wood pieces, coins, strings, nails and soil particles are removed. Later they are broken to loose their shells from the nibs and sieved into smaller number of fractions for optimum separation during the winnowing process. Lighter broken shells are removed by steam of air in the winnowing cabinet. Strong magnets are used to remove magnetic foreign materials from the nibs before further processing. The breaking and winnowing processes are highly important for separating the essential components of the bean (Afoakwa, 2010b).

2.2.3. Sterilization

Cocoa beans or nibs are exposed to high temperatures for a long time to inactivate all the microorganisms at the sterilization process. Factories perform this process before or after the roasting process depending on the factory and equipment used. All the microorganism that contaminate the cocoa nibs during the postharvest processes of fermentation, drying, packing and transportation are damaged in a batch or continuous process by wetting or heating with steam. Total Plate Count should be less than 500 per gram and all pathogenic bacteria should be destroyed. Nibs can be

directly roasted (natural process) or can be alkalized before roasting by the Dutch process. If the sterilization process will be performed after roasting, heat resistant bacteria and spores should be totally destroyed by the heat treatment that a fine water spray of steam into the roasting drum at the end of the roasting period of about 20 seconds (Awua, 2002).

2.2.4. Alkalization

Alkalization process is first designed by a Dutchman called Van Houten in 1928 and therefore, the process named as the Dutch process. All the cocoa, liquor, beans and nibs that undergo this process is called 'alkalized' or 'Dutched' (ADM Cocoa, 2006). Alkalization is the treatment of cocoa nibs with an alkali solution like potassium or sodium carbonate. This treatment increases the pH of the cocoa from 5.2-5.6 to 6.8-7.5 and makes it nearly neutral. Cocoa powder or cocoa liquor color and flavor can be modified according to the type of alkali used for alkalization process. It also enables the cocoa powder and cocoa liquor to have increased dispersibility in water. Alkali solution is sprayed into the drum, filled with the cocoa nibs and then slowly dried at 100°C (Awua, 2002; Afoakwa, 2010b). This process step is very important, because it causes markedly decreased procyanidin content and antioxidant capacity in cocoa processing (Gu et al., 2006).

2.2.5. Roasting

Roasting process is performed to develop original cocoa flavor that exist in the form of precursors during the fermentation and drying of the beans. Some physical and chemical changes during roasting of the fermented beans are; loosening of shells, moisture loss from the beans to about 2% final content, darkening of the nib color and resulting in more friable nibs. Besides, reduction in the number of microorganisms enables to produce additional food-grade products such as cocoa butter, cocoa liquor and cocoa powder. Moreover, denaturation of proteins and degradation of amino acids take place during roasting process. Natural reducing sugars are destructed during degradation of amino acids. Additionally, volatile acids and other substances that contribute to acidity and bitterness are decreased. Large number of volatile compounds has been detected such as alcohols, esters, aldehydes, ketones and pyrazines. There are very little changes for fats, polyphenols and alkaloids (Minifie, 1999).

Roasting temperature, time and rate of moisture loss directly affects the degree of changes during the process (Awua, 2002). According to the type of roasting, temperature varies between 90°C and 170°C. Cocoa processing industry uses three main kinds of roasting techniques. These are whole bean roasting, nib roasting and liquor roasting (Afoakwa, 2010b).

In whole bean roasting process, beans are roasted before winnowing process to remove shells. The disadvantage of this process is that it causes fat migration into the shells and therefore results in a loss of cocoa butter. In nib roasting process, many of the limitations of whole bean roasting are defeated by removing the shells before roasting. Flavor development becomes possible in nib roasting process by treating the nibs with alkaline or sugar solution in certain types of cocoa. In liquor roasting, thermal pre-treatment is used and the nib is ground to liquor before roasting. Main disadvantage of this process is that the shell must be removed before winnowing and it results in poor separation for certain cocoa types. Machines that evaporate the internal moisture by developing a high surface temperature forming pressure within the bean to remove the nib away from the nib are used (Afoakwa, 2010b).

2.2.6. Nib grinding and liquor treatment

Cocoa liquor is formed during the nib grinding process by milling of cocoa nibs. The aim of this process is to form smooth cocoa powder and chocolate taste with a minimum viscosity following the use of cocoa liquor. Fine grinding is essentially important for production of cocoa powder. The nib has about 55% cocoa butter content in solid form within the cells and grinding of nib cells to a particle size of 30 μm releases cocoa butter into liquor (Afoakwa, 2010b).

Cocoa butter melts to form cocoa liquor by heat treatment applied during the multistage grinding process. Aging and microbial inactivation performed in storage tanks by heating the cocoa liquor to about 90-100˚C after the cocoa liquor packaged for sale (Awua, 2002). Nearly 78-90% of cocoa butter is released during pressing and the residual lipids can be removed by supercritical fluid extraction (Beckett, 2000).

2.2.7. Liquor pressing

Cocoa butter is removed from the cocoa liquor by using hydraulic presses that applies high pressure of 520 kg/cm^2 and larger presses take a charge of 113.4 kg per

pressing cycle. At the end of this process cocoa cake of 10-24% fat content is produced according to pressing time and properties. High-fat containing cake includes 22-24% residual fat and low-fat containing cake includes 10-12% residual fat in the pressed cake. Extracted cocoa butter is poured into receptacles to be pumped into an intermediate tank for continual processes (Afoakwa, 2010b).

2.2.8. Cake grinding

The cake released from the liquor pressing process is in big size to be used in cocoa powder production. Therefore, in cake grinding process the aim is to break down the cocoa cakes into smaller pieces by kibbling machines and the product is called the kibbled cake. It may be blended before pulverization according to its fat content and degree of alkalization to produce a high quality cocoa powder (Afoakwa, 2010b).

2.2.9. Cocoa powder production

Kibbled cocoa cake particles are pulverized into cocoa powder with a desired level of fineness by using grinding lines including hammer-and-disk or pin mills. The fat of the cocoa powder crystallizes into a stable form by cooling after pulverization to prevent any discoloration like fat bloom or formation of lumps in the packages (ADM Cocoa, 2006). The flowing cocoa powder is then sieved and passed through magnets before packaging.

2.3. Chocolate Processing and Technology

Chocolate manufacturing process generally includes 5 main steps (Afoakwa, 2010b);

(1) Mixing
(2) Refining
(3) Conching of chocolate paste
(4) Tempering
(5) Moulding and demoulding

Processing steps are shown in Figure 2.1 for chocolate manufacture.

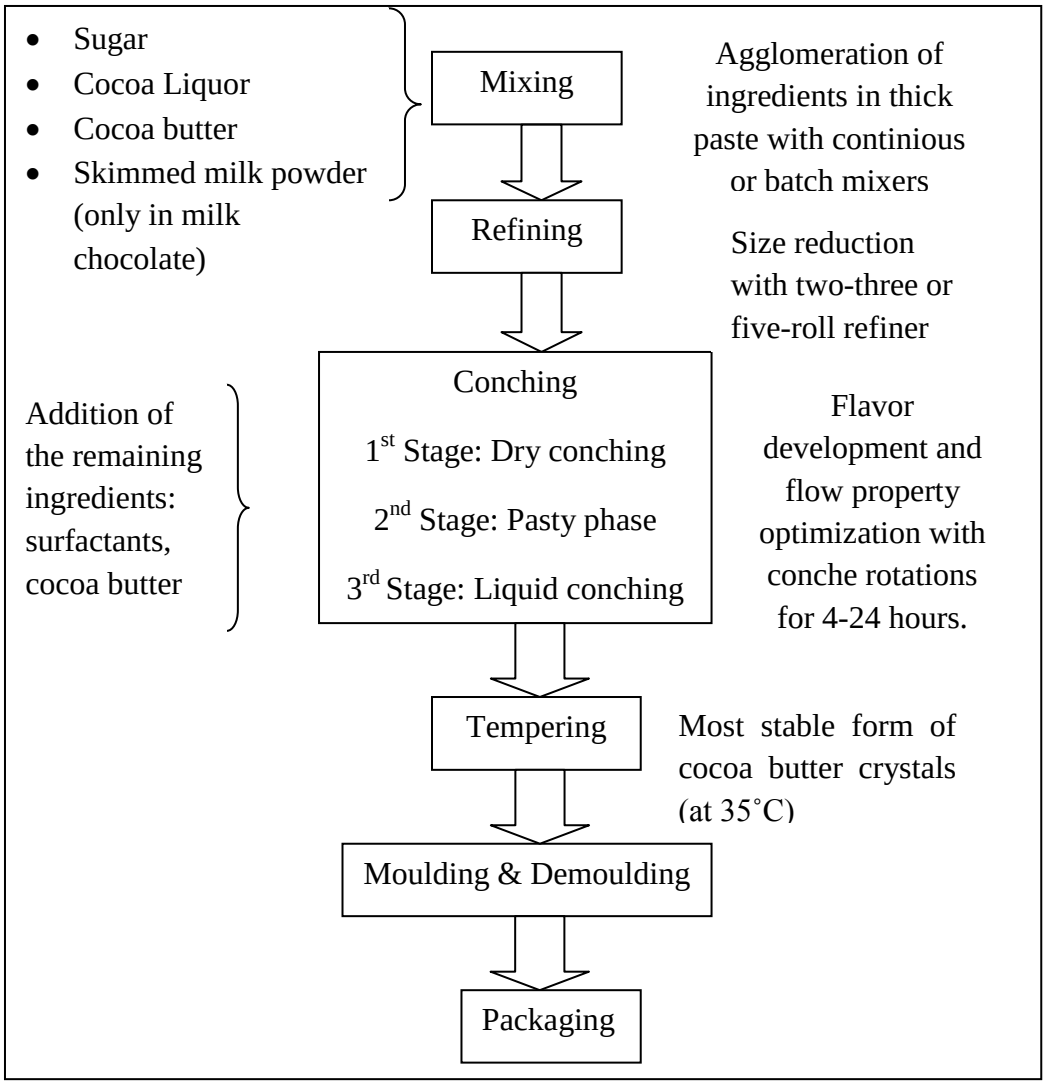

Figure 2.1 : Processing steps of chocolate manufacture (Afoakwa et al., 2007).

2.3.1. Mixing

Mixing is an essential process in which the ingredients are mixed in continuous or batch mixers using appropriate time-temperature combinations to form homogeneous product formulations. Depending on the desired chocolate recipe, cocoa liquor, sugar, cocoa butter, milk fat and milk powder is mixed for 12-15 minutes at 40-50˚C in batch mixers. Besides, in continuous mixing well-known automatic kneaders are used to produce chocolate products with tough texture and plastic consistency by large chocolate manufacturers like Cadbury and Nestle (Minifie, 1999; Beckett, 2000; Awua, 2002).

2.3.2. Refining

In modern chocolate industry, refining is an important process to obtain chocolate products with smooth texture. Depending on the type of chocolate particle size is decreased under a level of 30μm using two or five cylinder refiner combinations (Ziegler and Hogg, 2009). Rheological and sensory properties are directly related with the final particle size. Temperature is controlled by the internal water flow with hydraulic pressure. Knife blades are used to remove the thin chocolate film from the rollers. Optimum particle size is lower than a maximum value of 35 μm for dark chocolate but it varies according to the product compositions (Awua, 2002). Refiners not only affect the particle size and agglomerate breakdown but also help coating of the each particle surface with lipid. Therefore, the lipid coated solid particles absorbs volatile flavor compounds from cocoa (Afoakwa, 2010b).

2.3.3. Conching

There are two important reasons which are flavor development and flow property optimization that makes the conching process essential for the chocolate manufacturing process. The flavor of the chocolate depends upon a series of processes but the conching process is the final opportunity to obtain a fine flavor for a particular chocolate product. Cocoa mass has a very acidic flavor according to fermentation, drying and roasting processes. The objective of conching is removal of these distasteful flavors, development of the more desirable flavors and transfer flavor components between the ingredients. It is essentially important for dark chocolate and depends on the conche ventilation and conching time (Beckett, 2009).

In chocolate manufacturing plants the conche includes a hammer mill or a roll refiner to grind the chocolate mass to produce a chocolate paste or powder. The function of conche is to turn this into a flowable liquid that can be moulded or poured over the product center. Fat makes up about one thirds of the weight of chocolate and it melts in high temperature conditions. This enables the fat to flow into moulds and have a smooth texture in the mouth. If the surfaces of the sugar are uncoated by fat, it disables the flow of the chocolate. Conching process helps the fats to coat these surfaces and enables the flow of the chocolate. Mixing property of the conche breaks up agglomerates that are formed due to presence of moisture or amorphous sugar on the sugar surface (Beckett, 2009).

Conching time depends on the initial cocoa flavor intensity and the product in which it is being used. It is possible to shorten the conching time by pre-treatment of some of the acidic components (Beckett, 2009).

2.3.4. Tempering

Chocolate tempering is a thermal process that highly stable fat crystals of the chocolate are formed in a correct size and type homogeneously dispersed in the chocolate mass. It is a technique of controlled crystallization that is necessary for producing most stable solid form of cocoa butter in the finished product. During the cooling process these fat crystals form a micro-homogeneous solid fat crystal network following the moulding process. Liquid chocolate mass is tempered for long-term stability of the chocolate flow properties moulding and demoulding conditions. Another important effect of tempering is adjustment of the viscosity and yield value for moulding and demoulding. Final product will have following properties: good shape, color, gloss, away from mould, controlled weight, stable, harder, more heat resistant and longer shelf life. Untempered chocolate is soft and not effectively demoulded (Windhab, 2009).

Tempering has four main steps for crystallization of a small proportion of triglycerides with crystals forming nuclei (1-3% total) for remaining lipid to set in the correct form. First step is cooling to completion at 50˚C, second step is to cooling to point of crystallization at 32˚C, third step is crystallization and the fourth step is conversion of any unstable crystals at 29-31˚C (Talbot, 2009). Tempering sequence during lipid crystallization is shown in Figure 2.2.

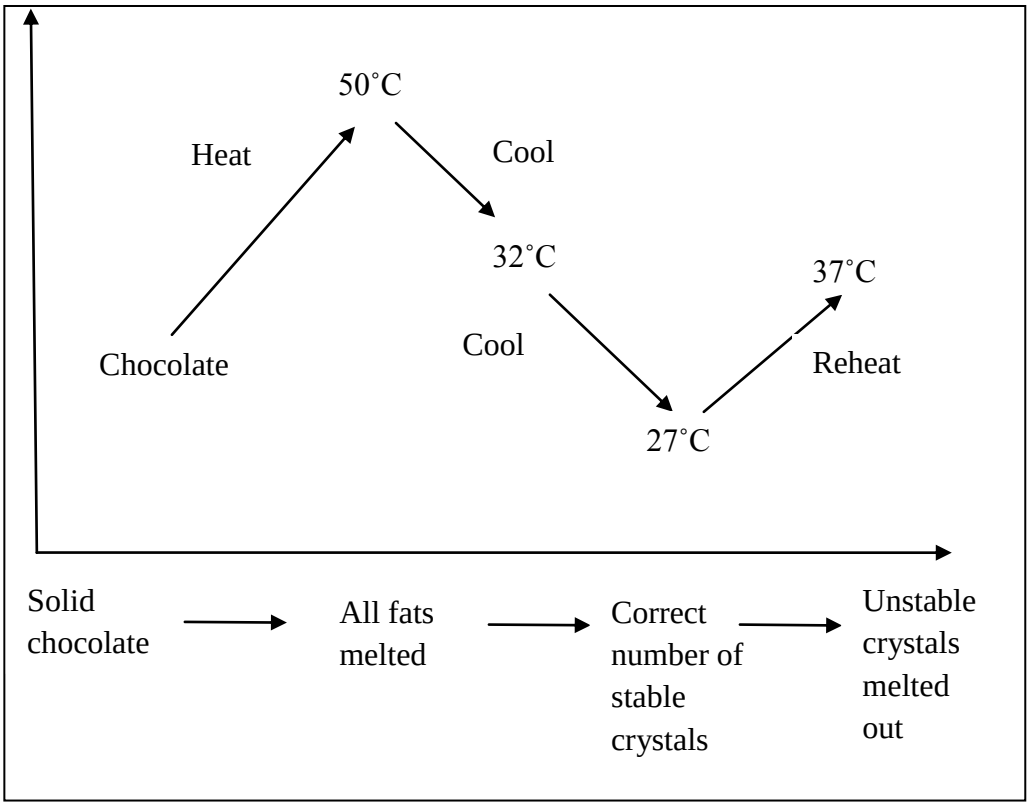

Figure 2.2 : Tempering sequence during lipid crystallization in chocolates (Afoakwa, 2010b).

Tempering process is closely related with the function of recipe, equipment and the final purpose. The tempering properties of milk chocolate are different from dark chocolate according to the milk fat molecules on crystal lattice formation (Haylock and Dodds, 2009).

2.3.5. Moulding, enrobing and cooling

Moulding and enrobing processes are generated to form finished chocolate product ready for demoulding. Moulding is a method that tempered chocolate product is poured into moulds to give shape. It is a simple way of dosing operation. Another way of moulding is that of forming a shell of chocolate and adding fillings into it. Enrobing is another method that pre-formed centre is coated with chocolate. Thickness and form of the chocolate product is controlled by blowing air and then vibrating. Moulding gives a more glossy appearance to chocolate product when it is compared with enrobing. Good tempering is crucial for enrobing process. In both methods, cooling is necessary for immediate wrapping of the chocolate produced.

Polycarbonate plastic moulds are used instead of metal ones to reduce noise in the production area. Moulds should be at the same temperature (+/- 1˚C) with the tempered chocolate. If the moulds are too warm, chocolate will be detempered and stick to the mould resulting in hard demoulding, poor glossy appearance and bloom. If the moulds are too cold, chocolate products will be less glossy and sticky (Gray, 2009).

2.4. Phenolic Compounds of Cocoa and Chocolate

2.4.1. Classification and chemical structure of phenolic compounds

Phenolic compounds are the secondary metabolites with a chemical structure of a hydroxyl group bonded to an aromatic ring that are not involved in growth and energy metabolism (Harnly et al., 2007). Phenolic compounds, with many subclasses like more than 450 flavonols, 400 flavones, 350 flavanones, 300 isoflavones, 19 anthocyanidins and 350 chalcones, represent the largest class of nonnutrients found in the plant kingdom. As a result of extensive glycosilation of the aglycone backbones and acylation of the glycosides large variety of subclasses are formed (Cuyken and Claeys, 2004). Phenolic compounds can be classified into two groups: basic phenolic compounds and polyphenols (Vermerris and Nicholson, 2006).

Polyphenols constitute one of the most numerous and widely distributed groups of substances in the plant kingdom that are large, structurally diverse group of organic compounds that contain multiple phenol functional groups (Manach et al., 2004). More than 8000 phenolic structures are known and dietary polyphenols represent the main source of antioxidant for human use (Bravo, 1998; Graf et al., 2005). Cocoa beans are rich source of polyphenols and dark chocolate is considered as one of the major antioxidant source after fruits and vegetables (Rusconi and Conti, 2010). Cocoa seeds are inedible because of the high concentration of polyphenols resulting in bitter flavor (Thomas-Barberan et al., 2007). Polyphenols in cocoa beans are placed in the pigment cells of cotyledons. The color of cocoa bean polyphenol storing pigment cells is ranged from white to purple according to the amount of anthocyanins. There are three main groups of polyphenols in cocoa and cocoa products: catechins or flavan-3-ols, anthocyanins and proanthocyanidins (Rusconi and Conti, 2010).

Polyphenolic compounds are divided into four classes: phenolic acids, stilbenes, lignans and flavanoids (Manach et al., 2004). The phenolic acids are carboxylic acids derived from benzoic or cinnamic acid skeletons. Stilbenes have a diphenylethane skeleton, and lignans are composed of two linked phenylpropane units. Flavonoids are composed of two phenyl rings linked by a propane bridge to form an oxygenated heterocyclic ring with a benzo-γ-pyrone structure, resulting in the characteristic 15 carbon (C6-C3-C6) flavan skeleton with three rings. The C6-C3-C6 flavan skeleton characteristic of flavonoids is given in Figure 2.3. The A- and B-rings are benzenes, and the C-ring is the central pyran heterocycle. (Beecher, 2003; Heim et al., 2002; Iwashina, 2000; Pietta, 2000; Yao et al., 2004).

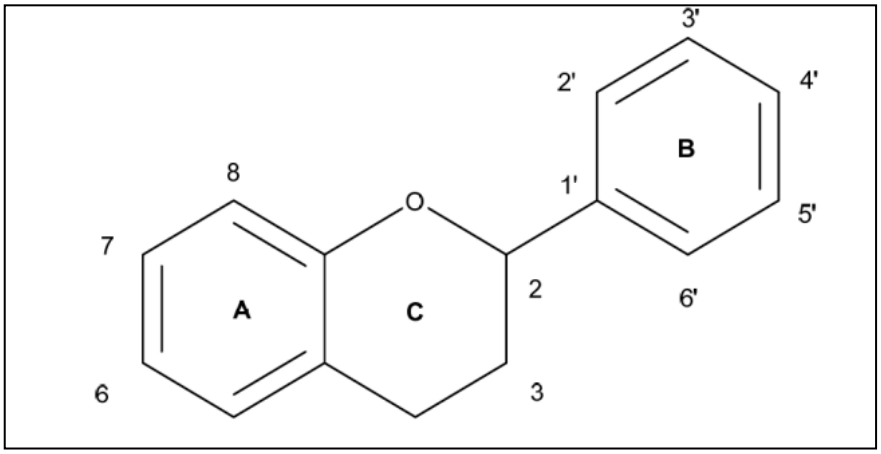

Figure 2.3 : The C6-C3-C6 flavan skeleton characteristic of flavonoids (Shahidi and Nazck, 2004)

Flavonoids have six major classes that are anthocyanins, flavonols, flavan-3-ols, flavanones, flavones and isoflavones. Minor classes of flavonoids include the chalcones, various types of isoflavonoids, flavan-4-ols, flavan-3,4-diols, aurones, biflavonoids, and oligoflavonoids (Shahidi and Nazck, 2004). The structures of six major sub-classes of flavonoids are given in Figure 2.4 Flavan-3-ols are the predominate class of flavanoid found in cocoa and chocolate.

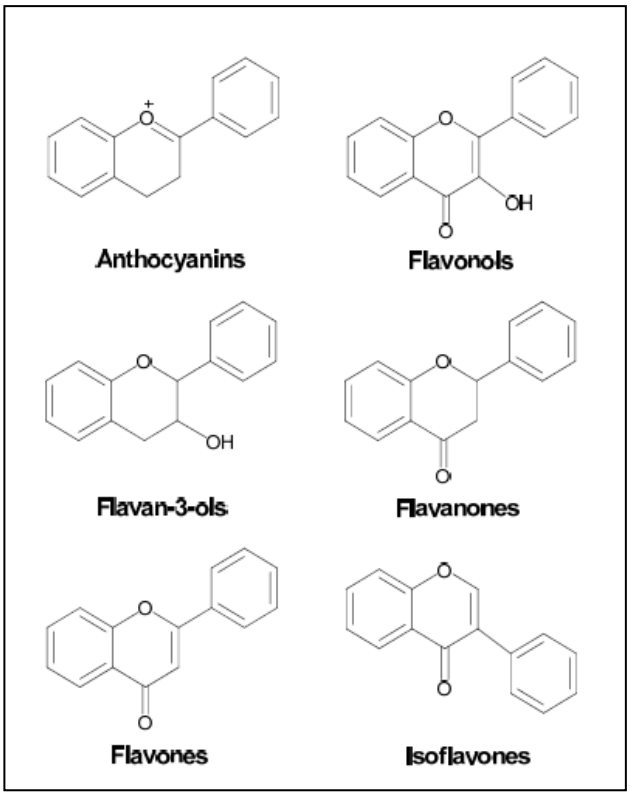

Figure 2.4 : The structures of six major sub-classes of flavonoids (Shahidi and Nazck, 2004).

Cocoa flavonoids are characterized as flavan-3-ols or flanavols and include the monomeric forms of (+) catechin and (-) epicatechin, and the complex oligomeric forms of the monomeric units named as procyanidins (Engler and Engler, 2004).

Catechins are monomeric flavan-3-ols or flavanols. They have a basic flavan skeleton with hydroxyl groups at C5 and C7 on the A-ring, at C3 on the C-ring, and at C3' and C4' on the B-ring. The various catechins differ in that certain species (gallocatechins) have a third B-ring hydroxyl group at C5', and certain species (catechin gallates) have a gallic acid residue esterified to the C3 hydroxyl group. Due to the presence of two chiral carbons in the C-ring (C2 and C3), multiple stereoisomers exist for each catechin structure. If the flavan skeleton is with the A-ring on the left and the B-ring on the right, the stereochemistry of C3 is described as (+) if the hydroxyl group is oriented up and (−) if the hydroxyl group is oriented down. If the orientation of the C2 substituent (the B-ring) and the C3 hydroxyl group is *trans* with respect to the plane of the C-ring, then they are referred to as simply catechins, whereas catechins with the *cis* orientation are referred to as epicatechins. The major catechins found in the human diet are: (+)-catechin, (−)- epicatechin, (−)-

epigallocatechin, (−)- epicatechin gallate, and (−)-epigallocatechin gallate (Del Rio et al., 2004; Inoue et al., 2002; Miketova et al., 1998; Miketova et al., 2000; Zeeb et al., 2000). The structures of these catechins and their epimers are shown in Figure 2.5.

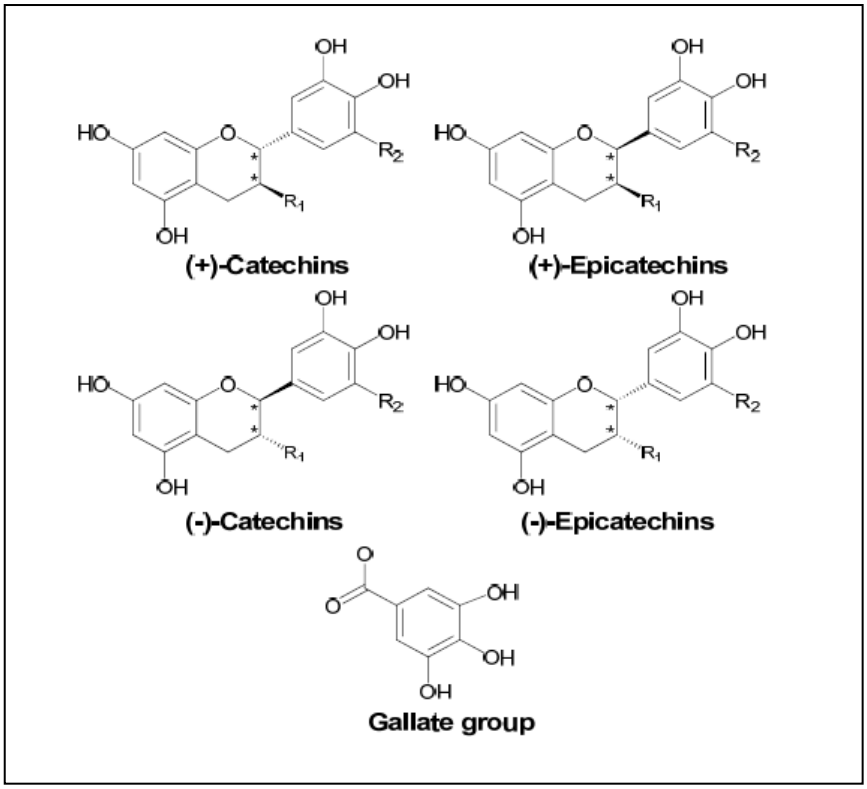

Figure 2.5 : Major flavan-3-ols in the diet (Shahidi and Nazck, 2004).

In addition to the various classes and subclasses of monomeric polyphenols, there exist more complex compounds composed of one or more polyphenol monomers that may also contain non-polyphenol residues such as sugars. One such group of compounds is the tannins. There are four subclasses of tannins: hydrolyzable tannins (gallotannins and ellagitannins), complex tannins, derived tannins (theaflavins), and condensed tannins (Khanbabaee and Van Ree, 2001). The condensed tannins are also called proanthocyanidins which constitute an important part of cocoa and chocolate polyphenols. They undergo oxidative depolymerization when heated in strongly acidic conditions, resulting in the release of anthocyanin monomers (Ferreira and Li, 2000; Jeong and Kong, 2004; Li and Deinzer, 2007).

The condensed tannins are dimers, oligomers (3-7 monomer residues), and polymers (8 or more monomer residues) composed of flavan-3-ol monomer residues (Monach et al., 2004; Beecher, 2003; Heim et al., 2002; Jeong and Kong, 2004; Li and

Deinzer, 2007). The degree of polymerization (DP) can range from 2 to greater than 50, although the mean DP in most plant tissues and foods is unknown (Monach et al., 2004; Khanbabaee and Van Ree, 2001).

The monomers of condensed tannins are typically bonded together by interflavan linkages between the C-ring of the first monomer and either the A- or C-ring of the next monomer. The B-type condensed tannins have only one interflavan linkage between adjacent monomer residues, which is typically a C4→C8 (or, less commonly, a C4→C6) carbon-carbon bond. The A-type condensed tannins, on the other hand, also include adjacent monomers joined by two interflavan linkages: the C4→C8 carbon-carbon bond plus a C2→O→C7 ether bond (Monach et al., 2004; Beecher, 2003; Heim et al., 2002; Khanbabaee and Van Ree, 2001; Ferreira and Li, 2000; Jeong and Kong, 2004; Li and Deinzer, 2007; Ferreira and Slade, 2002). The interflavan linkages may have either α or β configuration with respect to the A-C ring system of the first monomer, although the β configuration appears to predominate (Heim et al., Ferreira and Li, 2000; Ferreira and Slade, 2002). The C-type condensed tannins are simply trimeric B-type condensed tannins, and as such, this classification is of limited utility and is rarely mentioned (Khanbabaee and Van Ree, 2001).

The condensed tannins may include any of the flavan-3-ol momoners, and therefore the stereochemistry of the C2 and C3 carbons, as well as the substituents on C3 (-OH or -Gallate) and C5' (-H or –OH) depend on the nature of each precursor monomer. The monomer composition of a given condensed tannin may be either homogenous (containing only one type of monomer) or heterogeneous (containing multiple types of monomers). Condensed tannins that contain primarily (−)-epicatechin and (+)-catechin yield cyanin upon depolymerization, and are therefore referred to as the procyanidins (Beecher, 2003; Heim et al., 2002; Jeong and Kong, 2004). Due to the large number of possible constituent monomers, variable degree of polymerization and stereochemistry considerations, there are thousands of possible structures for condensed tannins, of which several hundred have been characterized in foods and other plant tissues (Monach et al., 2004; Jeong and Kong, 2004). Some common condensed tannins are: Procyanidin B1 [(−)-epicatechin-(4β→8)-(+)-catechin], Procyanidin B2 [(−)-epicatechin-(4β→8)-(−)-epicatechin], Procyanidin B3: [(+)-catechin-(4α→8)-(+)-catechin] Procyanidin A1 [(−)-epicatechin-(4β→8,2β→O→)-(+)-catechin], Procyanidin A2 [(−)-epicatechin-(4β→8,2β→O→7)-(−)-epicatechin],

Procyanidin C1 [(−)-epicatechin-(4β→8)-(−)-epicatechin-(4β→8)-(−)-epicatechin] (Jeong and Kong, 2004).

2.4.2. Composition of cocoa and chocolate polyphenols

Cocoa and chocolate are rich dietary source of catechin and catechin derivatives. The main catechin is (−)-epicatechin with up to 35% of polyphenol content. The difference from tea is that cocoa mostly contains non-gallated catechins which are catechins and epicatechins, while tea contains large quantities of gallated and/or gallocatechins. Besides monomers, cocoa and chocolate contain large quantities of procyanidins with a variety of degrees of polymerization (Adamson et al., 1999; Nelson and Sharpless, 2003; Sanchez-Rabaneda, 2003).

Phenolic compounds constitute 12-18% of the total weight of the dried and roasted cocoa beans. Roughly, 35% of the total polyphenols in non-fermented cocoa nibs of the *Forastero* variety is epicatechin. Different varieties of cocoa nibs have epicatechin content range between 34.65 mg/g and 43.27 mg/g for defatted samples. This amount decreases at fermentation and drying steps. (Wollgast and Anklam, 2000).

In addition to flavan-3 ols, catechin and epicatechin and their dimers procyanidin B1 and procyanidin B2 which are the major phenolic compounds in cocoa and chocolate, they contain some other polyphenols that have been identified and quantified. These polyphenols are: procyanidin B3 = catechin-(4α→8)-catechin, procyanidin B4 = catechin-(4α→8)- epicatechin, procyanidin B5 = epicatechin-(4β→6)-epicatechin, procyanidin C1 = epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin, procyanidin D= epicatechin-(4β→8)-epicatechin- (4β→8)-epicatechin-(4β→8)-epicatechin.

Cocoa and chocolate polyphenols also include higher oligo- and polymers, mostly homologues of epicatechin with 2–18 monomeric units, as well the following flavanols: quercetin, quercetin-3-O-glucoside (isoquercitin), quercetin-3-O-galactoside (hyperoside), quercetin-3-O-arabinoside. Moreover following flavones have been detected: apigenin, apigenin-8-C-glucoside (vitexin), apigenin-6-C-glucoside (isovitexin), luteolin and luteolin-7-O-glucoside, dihydroquercetin, dihydroxykaempferol, kaempferolrutinoside, naringenin, naringenin-glucoside, myricetin-glucoside.

The following phenolic acids are also identified: caffeic acid, chlorogenic acid, coumaric acid, ferulic acid, phenylacetic acid, phloretic acid, protocatechuic acid, syringic acid, vanillic acid, clovamide and dideoxyclovamide (Wollgast and Anklam, 2000; Ortega et al., 2008; Sanchez-Rabenada et al., 2003; Borchers et al., 2000).

Cocoa powder contains 0.7-2 mg/g catechin, 2-15 mg/g epicatechin and 25-55 mg/g total procyanidins on a free-fat basis (Gu et al., 2006; Miller et al., 2006; Natsume et al., 2000). Alkalized cocoa powder contains lower contents of catechin (0.3-0.4 mg/g), epicatechins (0.2-0.5 mg/g) and procyanidins (8-13 mg/g) when compared to natural cocoa powder (Gu et al., 2006). Dark chocolate have a higher content of flavanols with amounts of 0.15-0.5 mg/g catechin, 0.7-2 mg/g epicatechin and 0.5-31 mg/g procyanidin contents. Milk chocolate contains lower content of flavanols when compared to dark chocolate as a result of smaller content of % cocoa powder by weight than dark chocolate due to the addition of milk. Milk chocolate has lower levels of catechin (0.07-0.2 mg/g), epicatechins (0.3-0.4 mg/g) and procyanidins (0.6-3.2 mg/g) when compared to dark chocolate (Adamson et al., 1999; Gu et al., 2006; Miller et al., 2006; Natsume et al., 2000). It can be explained by milk protein interactions. Milk based products represent a very complex matrix that strong catechin-protein interactions are well-known to occur (Sibert et al., 1996). These protein-catechin interactions can directly interfere with accurate catechin determinations by significantly reducing analytical recovery (Komes et al., 2009).

Cocoa procyanidins mostly have degrees of polymerization from 2-10 (dimers through decamers) and larger dimers are present in smaller concentration (Adamson et al., 1999; Sanchez-Rabada et al., 2003; Gu et al., 2007; Miller et al., 2006; Natsume et al., 2000; Wollgast et al., 2001). The major procyanidins that are polymers of epicatechin in cocoa and chocolate are procyanidin B2 and procyanidin B5 which are dimers, procyanidin C1 which is trimer and cinnamtannin A2 is tetramer (Natsume at al., 2000; Cooper et al., 2007; Cooper et al., 2008). Porter et al. (1991) and later Hammerstone et al. (1999) identified oligomeric procyanidins built up mainly of epicatechin sub-units. Hammerstone et al. (1999) described procyanidins with up to twelve subunits (dodecamer) in unfermented cocoa.

Cocoa is different from majority of plant based foods as it contains significant amount of (−)-catechin whereas other contain (+)-catechin isomer. Natural *Thebroma cacao* beans also include (+) catechins as other plant based foods, but cocoa

fermentation and drying lead to the isomerization of (−)-epicatechin to form (−)-catechin. Therefore cocoa products may contain up 90% of (−)-catechin and only 10% of (+)-catechin according to some process steps (Gotti et al., 2006; Donovan et al., 2006).

There exists a great variation in both qualitative and quantitative profile of catechins and procyanidins in cocoa and chocolate products according to the differences in processing, formulation, geographical region and season.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Samples

Cocoa beans used in the choice of extraction solvent analysis were supplied by Altınmarka Food Industry & Trade Inc. All the chocolate samples used in total phenolic, total flavonoid, antioxidant activity and sensory analysis were produced at Şölen Chocolate Inc. pilot scale production laboratory in Gaziantep, Turkey.

3.1.2. Chemicals

All the chemicals and standards that were used in the analysis were: sodium carbonate (Merck), Folin-Ciocalteau's reagent (Merck and Sigma-Aldrich), sodium nitrite (Merck), aluminium chloride (Fluka), sodium hydroxide (Merck), potassium dihydrogen phosphate (Merck), dipotassium hydrogen phosphate (Merck), potassium persulfate (Riedel-de Haën), ABTS (AppliChem), methanol (analytical grade, Sigma-Aldrich), ethanol (analytical grade, Sigma-Aldrich), hexane (Emboy), copper (II) chloride (Merck), ammonium acetate (Merck), sodium acetate trihydrate (Merck), glacial acetic acid (Merck), hydrochloric acid (Merck), ferric chloride hexahydrate (Lachema), TPTZ (Merck), neocuproine (Roth), ABTS (AppliChem), DPPH (Fluka), Trolox (Sigma-Aldrich), (+)-catechin (Sigma-Aldrich).

3.2. Methods

3.2.1. Chocolate production at pilot scale plant

Chocolate production was performed at Şölen Chocolate Inc. pilot scale production laboratory in Gaziantep, Turkey. 5 kg of chocolate can be produced from each chocolate manufacturing process in this pilot scale production laboratory. Three different formulations were used for three different kinds of chocolate manufacturing processes;

- Dark chocolate with 65% cocoa and 15% almond content (Formulation A)
- Dark chocolate with 65% cocoa and 7.5% almond content (Formulation B)
- Dark chocolate with 65% cocoa content (Formulation C – control sample)

Deskinned almond paste was used in chocolate formulations A and B. The recipes of these three different kinds of chocolate manufacturing processes are given in Appendix A, Table A.1, Table A.2 and Table A.3, respectively. Production was repeated twice at the same pilot scale production laboratory and samples were taken from each step of the manufacturing processes for further analysis.

3.2.1.1. Mixing process

Ingredients were weighed according to each chocolate recipe and mixed about 15 minutes (Welbilt Ozti, Germany). At the end of mixing process samples were taken and labelled for further analysis.

3.2.1.2. Refining process

The mixed ingredients were taken from the mixer and placed in the refining machine. A three-roll refiner was used and temperature was controlled by internal water flow, held together by hydraulic pressure. A thin film of chocolate is attracted to increasingly faster rollers, travelling up to refiner until removed by a knife blade. Roller shearing fragments solid particles, coating new surfaces with lipids so that these become active, absorbing volatile flavor compounds from cocoa components. Refining cylinder (Bühler CH-9240 Uzmil, SDY 200, Germany) was used in the refining process. Temperature was 32°C and the hydraulic pressure was 11 bars. The hydraulic pressure that was applied to the knife should be 9 bars. Refining time has taken about 45 mins. Optimum particle size is 18 μm for dark chocolate and it was measured by micronmeter (Mitotuyo, USA) by droping sunflower oil on the final product of refining process that is called flex. At the end of refining process samples were taken and labelled for further analysis.

3.2.1.3. Conching process

The flex of the refining process was placed in the conche for conching process. Before adding the flex, 1 g of lecithin and 150 gr of cocoa butter were added into the conche and then flex was added on these. The Frisse conche is a typical example of an overhead conche used in modern chocolate industry. It consists of a large tank with three powerful intermeshing mixer blades, providing shearing and mixing action. Frisse pilot plant conche (Bühler Richard Frisse GmbH, D-32107, Germany) was used for conching process. Conching times and temperatures vary according to the types of chocolate. In pilot scale conching process of dark chocolate 70 °C temperature was applied for 6 hours. Conching was achieved in 11 steps according to the automatic computer program named El Kolino. The conching steps that were used by El Kolino program is given at Appendix B, Table B.1. Vanillin was added at the end of the conching step and also to give chocolate a suitable viscosity, additional cocoa butter and lecithin was added towards the end of conching to thin or liquefy the chocolate prior to tempering. At the end of each conching processes samples were taken and labelled for further analysis.

3.2.1.4. Tempering process

The conched chocolate was placed in the tempering machine for tempering process. Tempering machine (GAMI s.r.l, 36015, Italy) was used for pilot scale dark chocolate production. Chocolate was melted at 46 °C and cooled to point of crystallization at 32 °C. Tempering time has taken about 15 minutes. At the end of tempering processes samples were taken and labelled for further analysis.

3.2.1.5. Moulding and demoulding

After tempering process, the tempered chocolate was taken to a glass beaker and poured on the chocolate molds for moulding. The moulds should be cleaned with compressed air before moulding process. The temperature of the moulds should be at 30°C. After pouring chocolate on the moulds, the moulds were vibrated for giving appropriate shape to chocolate final products. The molds were put into the drying cabins (Vötsch Industrietechnic, VC 0034, Germany) at temperature 16 °C for 15-20 mins before demoulding. They were demoulded and packaged at the end. Samples were taken and labeled for further analysis.

3.2.2. Total phenolics, total flavonoids and antioxidant capacity of chocolate samples and their major ingredients

All the samples that were collected from the first and the second chocolate manufacturing processes at the pilot scale plant: mixing, refining, conching, tempering, chocolate final product samples and their ingredients alkalized cocoa powder, almond and cocoa mass were analyzed twice. The total phenolics, total flavonoids and antioxidant capacities according to ABTS (2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) method, DPPH (1,1-diphenyl-2- picrylhydrazyl) method, cupric reducing antioxidant capacity (CUPRAC) and ferric reducing antioxidant power (FRAP) analysis results were obtained.

3.2.2.1. Sample preparation

All the samples were prepared as described by Guyot et al. (1998), Hammerstone et al. (1999) and Komes et al. (2009) with some modifications. All the samples were stored at -80°C before starting to sample preparation procedure. The samples were grinded with nitrogen. They were freeze-dried for 22 hours. In order to eliminate lipids from the samples, they were extracted three times with hexane of five times more volume of the grams of samples. 10 grams of a sample was extracted with 50 ml of hexane for three times to remove lipids. The defatted solids were air-dried during 24 hours to remove the residual organic solvent (Adamson et al., 1999). The freeze-dried non-fat samples were weighed 2 g in centrifuge tubes. For all the samples three separate centrifuge tubes were prepared for triple analysis. The phenolic compounds were extracted in two stages from all the samples. 5 ml of 80% methanol solution was added on the 2 g weighed sample in a centrifuge tube. The centrifuge tubes were vortexed for 30 seconds and then they were sonicated in ultrasonic bath without any heat application for 15 minutes. The mixtures were centrifuged for 10 minutes at 3000 rpm and the supernatants were decanted. The upper layers were transferred to separate centrifuge tubes. Again 5 ml of 80% methanol solutions were added on the residue in centrifuge tubes. They were sonicated for 15 minutes and vortexed for 30 seconds. They were centrifuged for 10 minutes at 3000 rpm and the supernatants were decanted. The upper layers were

transferred to the extract containing centrifuge tubes and filled up to obtain 10 ml of extract. The centrifuge tubes containing the extracts were stored in a freezer at -18°C.

3.2.2.2. Determination of optimum solvent for the analysis of phenolic content, flavonoid content and antioxidant capacity

Cocoa beans were used for deciding the extraction solvent that is more effective to extract phenolics. 70% methanol solvent and 80% methanol solvent were used for extraction. Sample extracts were prepared and total phenolics, total flavonoids, antioxidant capacity assays of ABTS (2,2' azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) method, DPPH (1,1-diphenyl-2- picrylhydrazyl) method, cupric reducing antioxidant capacity (CUPRAC) and ferric reducing antioxidant power (FRAP) were performed.

3.2.2.3. Determination of total phenolic content

The total phenolics were measured colorimetrically as described by Shahidi and Naczk (1995) with slight modifications. Ten fold diluted Folin-Ciocalteu's Phenol Reagent with milli-Q water, 7.5% Na_2CO_3 dissolved in milli-Q water and catechin stock solution were prepared before analysis. Folin-Ciocalteu's phenol reagent should be kept in dark. A 50 µl diluted phenolic extract, a 1.5 ml of ten-fold diluted Folin-Ciocalteu's Phenol Reagent, 1.5 ml of 7.5% sodium carbonate solution were mixed in test tubes and all the tubes are vortexed well. Catechin was used as calibration reference standard, and calibration solutions contained 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9 mg/ml catechin in 80% methanol solution diluted from the same catechin stock solution (1 mg/ml). The calibration curve is shown in Appendix C, Figure C.1. Absorbances were measured against water at 765 nm after waiting for 30 minutes at room temperature in dark using Shimadzu UV-1700 Pharmospec UV-Visible Spectrofotometer. Instead of sample extract 80% methanol solutions were used and the absorbances were saved as blank. The measured blank absorbances were subtracted from sample and standard absorbances. All analyses were repeated for each sample extract three times. The content of phenolics was expressed as mg catechin equivalent (CE)/100 g dry weight.

3.2.2.4. Determination of total flavonoid content

The total flavonoids were measured colorimetrically as described by Dewanto et al. (2002), Zhishen et al. (1999) and Lee et al. (2003) with few optimised modifications. Prior to analysis 5% sodium nitrite ($NaNO_2$), 10% aluminium chloride hexahydrate ($AlCl_3.6H_2O$), 1 M sodium hydroxide (NaOH) and catechin stock solution (1 mg/ml) were prepared. Sodium nitrite was prepared by dissolving 5 g of $NaNO_2$ in 100 ml of Milli-Q water. Aluminium chloride hexahydrate was prepared by dissolving 10 g of $AlCl_3.6H_2O$ in 100 ml of Milli-Q water. This was done under fume-hood, by adding water very slowly. NaOH 1 M was prepared by dissolving 4 g of NaOH in 100 ml of Milli-Q water. A 1 ml of diluted samples was mixed with 0.3 ml of 5% $NaNO_2$. 5 minutes later 0.6 ml of 10% $AlCl_3.6H_2O$ was added. At sixth minute, 2 ml 1 M of NaOH were added. Immediately 2.4 ml of water were added on test tubes and vortexed. Catechin was used as calibration reference standard, and calibration solutions contained 0.025, 0.05, 0.01, 0.02, 0.03, 0.04 mg/ml catechin in 80% methanol solution diluted from the same catechin stock solution (1 mg/ml). The calibration curve is shown in Appendix C, Figure C.2. Absorbances were read against 80% methanol solution at 510 nm using Shimadzu UV-1700 Pharmospec UV-Visible Spectrofotometer without waiting against a reagent blank. All analyses were repeated for each sample extract three times. The content of flavonoids was expressed as mg catechin equivalent (CE)/100 g dry weight.

3.2.2.5. Determination of antioxidant capacity according to ABTS method

Anioxidant capacities of the samples were measured colorimetrically as described by Miller and Rice-Evans (1997) with few optimised modifications. Prior to analysis $ABTS^{\cdot+}$ solution, potassium persulfate ($K_2S_2O_8$) solution, 0.05 M potassium phosphate buffer of pH 8.0 and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) stock solution were prepared. $ABTS^{\cdot+}$ solution was prepared by dissolving 220 mg of $ABTS^{\cdot+}$ in 200 ml milli-Q water. Potassium persulfate was prepared by dissolving 38 mg of $K_2S_2O_8$ in 2 ml milli-Q water. These two potassium persulfate and $ABTS^{\cdot+}$ solutions were mixed together and stored overnight in the dark to complete radicalization. 0.05 M potassium phosphate buffer of pH 8.0 (KP_i) was prepared by mixing 0.05 M potassium dihydrogen phosphate (MW=136.06 g/mol) and 0.05 M dipotassium hydrogen phosphate (MW=174.18 g/mol) measuring

the pH with pH meter to reach pH 8.0. $ABTS^{\cdot+}$ solution was diluted with 0.05 M KPi buffer at pH 8.0 until its absorbance reached 0.9 ± 0.2. The pH of the mixture should be 7.4 at the end. A 100 µl of diluted sample extract was mixed with 1 ml of $ABTS^{\cdot+}$ solution with 0.05 M KPi buffer in test tubes and vortexed for 10 seconds. The absorbances were read against water at 734 nm using Shimadzu UV-1700 Pharmospec UV-Visible Spectrofotometer after 30 seconds. A 100 µl of 80% methanol solution was mixed with 1 ml of $ABTS^{\cdot+}$ solution with 0.05 M KPi buffer in test tubes before each triplicate analysis of samples and the absorbance was saved as blank. Trolox was used as calibration reference standard, and calibration solutions contained 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.1 mg/ml trolox in 80% methanol solution diluted from the same trolox stock solution (1 mg/ml). The calibration curve is shown in Appendix C, Figure C.3.The sample and standard absorbances were subtracted from the blank. All the analyses were repeated for each extract three times and the results were expressed as mg trolox equivalent (TEAC) values with respect to the standard for 100 g of dry weight.

3.2.2.6. Determination of antioxidant capacity according to DPPH method

Antioxidant capacities of the samples were measured colorimetrically as described by Kumaran et al. (2006) and Rai et al. (2006) with few optimised modifications. Prior to analysis 0.1 mM of DPPH solution was prepared in methanol by dissolving 0.0394 g/l. DPPH solution should be kept in dark. 100 µl of diluted sample extracts were mixed with 2 ml of 1 mM DPPH in methanol. They were vortexed for 10 seconds and stored in the dark at room temperature. The absorbances were measured at 517 nm against methanol using Shimadzu UV-1700 Pharmospec UV-Visible Spectrofotometer after 30 minutes. Instead of sample extracts 80% methanol solutions were used and the absorbances were saved as blank. Trolox was used as calibration reference standard, and calibration solutions contained 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.1 mg/ml trolox in 80% methanol solution diluted from the same trolox stock solution (1 mg/ml). The calibration curve is shown in Appendix C, Figure C.4. The sample and standard absorbances were subtracted from the blank absorbances. All the analyses were repeated for each extract three times and the results were expressed as mg trolox equivalent (TEAC) values with respect to the standard for 100 g of dry weight.

3.2.2.7. Determination of antioxidant capacity according to CUPRAC method

Antioxidant capacities of the samples were measured colorimetrically as described by Apak et al. (2004) with few optimised modifications. Prior to analysis copper (II) chloride solution at a concentration of 10^{-2} M, ammonium acetate (NH_4Ac) buffer at pH 7.0 and neocuproine (Nc) solution in ethanol at a concentration of $7.5x10^{-3}$ M were prepared. Copper (II) chloride solution at a concentration of 10^{-2} M was prepared by dissolving 0.8524 g of $CuCl_2.H_2O$ in 500 ml of Milli-Q water. Ammonium acetate buffer at pH 7.0 was prepared by dissolving 38.54 g NH_4Ac in 500 ml of Milli-Q water. Neocuproine solution in ethanol at a concentration of $7.5x10^{-3}$ M was prepared by dissolving 0.78 g of Nc in 500 ml of ethanol. Neocuproine solution should be prepared daily. 1 ml of copper (II) chloride solution, 1 ml of neocuproine solution, 1 ml of ammonium acetate buffer, 100 µl of sample extract or standard extracts for calibration curve and 1 ml of Milli-Q water were mixed, respectively in test tubes. The absorbances were measured at 450 nm against water using Shimadzu UV-1700 Pharmospec UV-Visible Spectrofotometer after 30 minutes. Instead of sample extract 80% methanol solutions were used and the absorbances were saved as blank. Trolox was used as calibration reference standard, and calibration solutions contained 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 mg/ml trolox in 80% methanol solution diluted from the same trolox stock solution (1 mg/ml). The calibration curve is shown in Appendix C, Figure C.5. The measured blank absorbances were subtracted from sample and standard absorbances. All the analyses were repeated for each extract three times and the results were expressed as mg trolox equivalent (TEAC) values with respect to the standard for 100 g of dry weight.

3.2.2.8. Determination of antioxidant capacity according to FRAP method

Antioxidant capacity of the samples were measured colorimetrically as described by Benzie and Strain (1996), Deighton et al. (2000) and Degl'innocenti et al. (2007) with few optimised modifications. Prior to analysis 300 mM sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) solution at pH 3.6, 20 mM ferric chloride hexahydrate ($FeCl_3.6H_2O$) solution, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution were prepared. Ferric chloride hexahydrate solution was prepared by dissolving 0.2702 $FeCl_3.6H_2O$ in 49 ml of Milli-Q water and adding 1 ml of 1M HCl. While weighing

ferric chloride hexahydrate metal containers cannot be used and this reagent should be prepared fresh immediately prior to procedure. TPTZ solution was prepared by adding 0.156 g TPTZ to 50 ml of ethanol. TPTZ solution should be stored in dark glass bottle at room temperature. Sodium acetate buffer was prepared by adding 3.1 g sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) and 16 ml of glacial acetic acid and brought total volume to 1 liter with Milli-Q water. Sodium acetate solution should be stored in glass bottle at room temperature. FRAP reagent was prepared by combining 10x volume of sodium acetate solution with 1x volume of TPTZ solution and 1x volume of sodium chloride solution. FRAP reagent must be prepared fresh immediately prior to procedure and is stable for at least 2 hours at room temperature. After preparing the FRAP reagent, 900 µl of FRAP reagent and 100 µl of diluted sample extract / standard Trolox were mixed in test tubes and vortexed quickly for 20 seconds. The absorbances were measured at 593 nm against water using Shimadzu UV-1700 Pharmospec UV-Visible Spectrofotometer after 4 minutes following the addition of FRAP reagent. Instead of sample extract 80% methanol solutions were used and the absorbances were saved as blank. Trolox was used as calibration reference standard, and calibration solutions contained 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.1 mg / ml trolox in 80% methanol solution diluted from the same trolox stock solution (1 mg/ml). The calibration curve is shown in Appendix C, Figure C.6. The measured blank absorbances were subtracted from sample and standard absorbances. All the analyses were repeated for each extract three times and the results were expressed as mg trolox equivalent (TEAC) values with respect to the standard for 100 g of dry weight.

3.2.2.9. Statistical analysis

All the experiments were carried out in triplicate. Data were reported as means ± standard deviation for triplicate determinations. Analysis of variance and significant difference test (Duncan's Multiple Range Test), with a significance level of α=0.05%, were conducted to identify differences among means using IBM SPSS Statistics software (version 19.0 for Windows, SPSS Inc, Chicago, IL.). ANOVA tables are given in Appendix D.

In addition, Pearson's correlation analysis between antioxidant capacity, total phenolic content and total flavonoid content of chocolate formulations in all

manufacturing steps was also performed. Besides, t-test analyses were performed using the same statistical package to elucidate if any significant diference existed between the ingredients used in each production experiment and results were given in Appendix F, Table F.1.

3.2.3. Sensory analysis

3.2.3.1. Samples

In sensory analysis, chocolate final product samples that were produced in Şölen chocolate production pilot plant were used. There were three different kinds of chocolate; dark chocolate with 65% cocoa content as a control sample, dark chocolate with 65% cocoa and 15% almond content and dark chocolate with 65% cocoa and 7.5% almond content.

3.2.3.2. Quantitative descriptive analysis (QDA)

QDA was carried out by a sensory expert panel. This panel was composed of 12 trained panelists from Istanbul Technical University Food Engineering Department. This panel was selected on the basis of their sensory performances and interest and trained for 6 hours to perform quantitative descriptive analysis. The panel was trained to understand and correctly apply the descriptive terms to decrease variation.

The quantitative descriptive analysis was carried out in the sensory laboratory at Istanbul Technical University Food Engineering Department equipped with a round table for training sessions and individual booths for sensory evaluation according to the international standards (ISO, 2010).

The language development step was carried out over 3 sessions. These sessions, each lasted for 2 hours were done to define the sensory terms and define each descriptive. Assessors tasted many different kinds of dark chocolate samples in order to obtain a great number of descriptors. Panel leader led a discussion to reach a consensus on the descriptors.

Twenty attributes were generated by trained panelists according to the method described in the ISO 13299:2003 (ISO, 2003). The panel form is given in Appendix E, Figure E.1. Chocolate samples were evaluated according to a completely randomized design to balance presentation order and carry over effect. For panel

performance, dark chocolate samples were evaluated in 2 different sessions in panel booths. Three kinds of packed samples were presented on a plastic white color plate and the panelists tasted the chocolate samples starting from left to right. Samples were labeled with three-digit random numbers. Assessors scored each attribute on a 0-7 category scale. Category scales were described by using the terms like ‘none’ for ‘0’ and ‘very much’ for ‘7’. During each session three different dark chocolate samples were evaluated for all the attributes and two replicates were obtained for all the assessors for every sample (3 variants x 2 replicates x 12 assessors).

3.2.3.3. Statistical analysis

Kruskal-Wallis test was used to analyze the sensory analysis data of two sensory panels to determine if there were significant differences ($P<0.05$) for predetermined descriptives between different chocolate samples tested by panelists using IBM SPSS Statistics software (version 19.0 for Windows, SPSS Inc., Chicago, IL.).

4. RESULTS AND DISCUSSION

The results of the effect of almond content on chocolate total phenolic contents, total flavonoid contents and antioxidant capacities were discussed by comparing three different formulations of the chocolate products in all production steps. The formulations were grouped as; chocolate with 65% cocoa and 15% almond content (A), chocolate with 65% cocoa and 7.5% almond content (B) and chocolate with 65% cocoa content (control, C).

In addition, the effects of each manufacturing process steps on chocolate formulations were interpretted by grouping the manufacturing process steps as mixing process, refining process, conching process, tempering process, and moulding and demoulding process to produce chocolate final products. The samples of different manufacturing steps were abbreviated as; MX for mixing process, RF for refining process, CO for conching process, TE for tempering process, LP for moulding and demoulding process which is the last step for producing chocolate last product. The numbers next to the abbreviations show the manufacturing numbers of the first and the second production.

Moreover, the effect of using different chocolate ingredients that were used in different productions was discussed. These chocolate ingredients are cocoa mass, alkalized cocoa powder and almond paste. The total phenolic content, total flavonoid content and antioxidant capacities of cocoa mass, alkalized cocoa powder and almond paste used in each chocolate production were compared to evaluate the differences between two independent processing events resulting from the ingredients. These ingredients were also compared with chocolate final products.

Finally, the sensory analysis results of three chocolate formulations were discussed for pre-determined descriptives of chocolate samples tasted by panelists. Statistically significant differences between three chocolate formulations were specified.

4.1. Solvent Choice for Spectrophotometric Methods

The results of the two solvent systems (70% methanol solvent and 80% methanol solvent) are given in Table 4.1.

Table 4.1 : Comparison of two solvent systems to determine total phenolics, total flavonoids and antioxidant capacities in cocoa beans[1].

	70% methanol solvent	**80% methanol solvent**
Total Phenolics (mg CE/100 g dry weight)	8776.27 ± 548.12	13310.02 ± 114.98
Total Flavonoids (mg CE/100 g dry weight)	1439.04 ± 83.79	2055.20 ± 30.96
ABTS (µmol TEAC/100 g dry weight)	28457.80 ± 3665.50	31325.58 ± 503.71
DPPH (µmol TEAC/100 g dry weight)	20022.44 ± 947.18	33198.21 ± 2603.86
CUPRAC (µmol TEAC/100 g dry weight)	29929.23 ± 1230.58	33622.24 ± 2455.74
FRAP (µmol TEAC/100 g dry weight)	6629.28 ± 52.69	9510.84 ± 284.26

[1]Values represent the means ± standard deviations of three measurements from three extracts.

According to the results, total phenolic contents of cocoa beans in 80% methanol extracts were 34% higher than that of 70% methanol extracts. Besides, total flavonoid contents of cocoa beans in 80% methanol extracts were 30% higher than that of 70% methanol extracts. ABTS and DPPH results of cocoa bean samples showed that 80% methanol extracts yielded with 9% and 39% higher values than 70% methanol extracts, respectively. Similarly, CUPRAC and FRAP results showed that 80% methanol extracts were 11% and 30% higher compared to the 70% methanol extracts.

It is known that obtaining higher yields of phenolic compounds can be possible by increasing the polarity of the extraction solvent (Cheung et al., 2003). Azizah et al. (1999) studied seven types of solvent systems and stated that methanol proved to be

the best solvent for extracting antioxidants from cocoa by-products. Sun and Spranger (2005) reported that 80% methanol is the best solvent both for catechins and oligomeric and polymeric procyanidins extraction. In the light of current literature and the results obtained in the study methanol was selected for the cocoa and chocolate extraction. These extracts were used for the analysis of total phenolics, total flavonoids and antioxidant capacity tests.

4.2. Phenolic Contents of Major Chocolate Ingredients

4.2.1. Total phenolic content

The total phenolic contents of the alkalized cocoa powder, cocoa mass and deskinned almond paste that were used in the chocolate manufacturing of each production were measured. Table 4.2 represents the total phenolic contents of major chocolate ingredients used in the first and the second production. The total phenolic contents of the first production and the second production were compared to evaluate the differences in phenolic contents of ingredients that were used in each production (Table 4.2).

Table 4.2 : Total phenolic contents of major chocolate ingredients used in the process[1].

INGREDIENTS	FORMULATION	1	2	3	mg CE/100 g	Average mg CE/100 g
ALKALIZED COCOA POWDER	A1	489.19	488.1	487.01	488.10	475.91 ± 9.57
	B1	466.24	471.49	465.59	467.77	
	C1	468.65	473.67	473.24	471.85	
	A2	547.55	544.49	547.33	546.46	544.76 ± 3.22
	B2	548.20	544.93	547.55	546.89	
	C2	542.08	539.24	541.43	540.92	
COCOA MASS	A1	1939.98	1935.61	1930.15	1935.25	1865.91 ± 108.14
	B1	1723.60	1727.97	1714.86	1722.14	
	C1	1924.68	1944.35	1952.00	1940.34	
	A2	1838.35	1820.86	1835,07	1831.43	1650.26 ± 244.28
	B2	1333.46	1314.89	1328.00	1325.45	
	C2	1800.10	1783.71	1797.91	1793.91	
DESKINNED ALMOND PASTE	A1	11.30	10.54	11.72	11.18	10.42 ± 0.95
	B1	9.95	9.21	9.81	9.66	
	A2	8.45	9.97	9.50	9.31	8.59 ± 0.96
	B2	8.14	7.40	8.05	7.87	

[1]Values represent the means ± standard deviations of 3 replicates from 3 extracts of 2 independent processing events.

All three formulations were evaluated together and the average total phenolic contents of the first production were obtained as 475.91 mg CE/100 g DM for alkalized cocoa powder, 1865.91 mg CE/100 g DM for cocoa mass and 10.42 mg CE/100 g DM for deskinned almond paste. The average phenolic contents of the second production were obtained as 544.76 mg CE/100 g DM for cocoa powder, 1650.26 mg CE/100 g DM for cocoa mass and 8.59 mg CE/100 g DM for deskinned almond paste (Table 4.2).

Paired sample t-test analysis was performed between the first and the second production of major chocolate ingredients to compare the effect of using different ingredients on total phenolic content. It was observed that the differences between the total phenolic contents of ingredients used in the first and the second production were found to be statistically significant ($P<0.05$) (Table F.1).

Lee at al. (2003) compared the total phenolic, total flavonoid and antioxidant capacities of cocoa, black tea, green tea and red wine and found that cocoa contained higher levels of total phenolics (611 mg GAE/serving) and flavonoids (564 mg epicatechin equivalents/serving).

Total phenolic contents of alkalized cocoa powder, cocoa mass and deskinned almond paste were compared with chocolate final products in following section 4.3.

4.2.2. Total flavonoid content

The total flavonoid contents of the alkalized cocoa powder, cocoa mass and deskinned almond paste that were used in the chocolate manufacturing process were measured. The total flavonoid contents of the first production and the second production were compared to evaluate the differences in total flavonoid contents of ingredients that were used in each production (Table 4.3).

All three formulations were evaluated together and the average total flavonoid contents of the first production were observed as 304.17 mg CE/100 g DM for alkalized cocoa powder, 1713.38 mg CE/100 g DM for cocoa mass and 7.57 mg CE/100 g DM deskinned almond paste. The average phenolic contents of the second production were observed as 348.46 mg CE/100 g DM for cocoa powder, 1511.05 mg CE/100 g DM for cocoa mass and 6.61 mg CE/100 g DM for deskinned almond paste (Table 4.3).

Table 4.3 : Total flavonoid contents of major chocolate ingredients used in the process[1].

INGREDIENTS	FORMULATION	1	2	3	mg CE / 100 g	Average mg CE / 100 g
ALKALIZED COCOA POWDER	A1	305.45	294.26	303.7	301.14	304.17 ± 6.69
	B1	312.10	315.24	305.45	337.15	
	C1	300.21	296.71	304.4	310.93	
	A2	339.02	334.12	338.32	363.73	348.46 ± 12.11
	B2	365.59	360.35	365.24	300.44	
	C2	346.36	341.82	345.31	344.5	
COCOA MASS	A1	1751.46	1747.96	1744.46	1747.96	1713.38 ± 31.84
	B1	1682.23	1668.24	1678.03	1676.17	
	C1	1706.00	1715.09	1726.98	1716.03	
	A2	1659.15	1649.36	1656.35	1654.95	1511.05 ± 168.26
	B2	1294.12	1284.33	1292.02	1290.15	
	C2	1592.72	1580.83	1590.62	1588.05	
DESKINNED ALMOND PASTE	A1	8.34	7.78	8.66	8.26	7.57 ± 0.89
	B1	6.24	7.36	7.01	6.87	
	A2	7.5	6.94	7.4	7.28	6.61 ± 0.79
	B2	6.14	5.58	6.07	5.93	

[1]Values represent the means ± standard deviations of 3 replicates from 3 extracts of 2 independent processing events.

Paired sample t-test analysis was performed between the first and the second production of major chocolate ingredients to compare the effect of using different ingredients on total flavonoid content. It was observed that the differences between the total flavonoid contents of ingredients used in the first and the second production were found to be statistically significant ($P<0.05$) (Table F.1). Additionally, total flavonoid contents of alkalized cocoa powder, cocoa mass and deskinned almond paste were compared with chocolate final products in following section 4.3.

Alkalized cocoa powder was used as an ingredient of chocolate production. According to Andres-Lacueva et al. (2008), the alkalization process of the cocoa resulted in 60% loss of the mean total flavonoid content. (-)-Epicatechin content resulted in a larger decline (67%) than (+)-catechin (38%) according to its epimerization into (-)-catechin which is a less bioavailable form of catechin. Flavonol content also decreased, quercetin presented the highest loss (86%), whereas quercetin-3-glucuronide, quercetin-3-arabinoside, and isoquercitrin showed similar

decreases (58, 62, and 61%, respectively). These decreases also affected the antioxidant properties and the polyphenol bioavailability of alkalized cocoa powder products. Natural cocoa presented a mean content of total flavonoids 2653.13 µg/g (-)-epicatechin whereas alkalized cocoa powder presented 1055.37 µg/g (-)-epicatechin equivalents (Andres-Lacueva et al., 2008).

4.2.3. Total antioxidant activity

4.2.3.1. ABTS

Average total antioxidant capacities of major chocolate ingredients are shown in Table 4.11. All three formulations were evaluated together and the average total antioxidant capacities of the first production were obtained as 4134.69 µmol TEAC/100 g DM for alkalized cocoa powder, 18507.60 µmol TEAC/100 g DM for cocoa mass and 220.46 µmol TEAC/100 g DM for deskinned almond paste. The average total antioxidant capacities of the second production were obtained as 4317.07 µmol TEAC/100 g DM for cocoa powder, 16549.38 µmol TEAC/100 g DM for cocoa mass and 211.51 µmol TEAC/100 g DM for almond paste (Table 4.4).

Table 4.4 : Total antioxidant capacities of major chocolate ingredients used in the process according to ABTS method[1].

INGREDIENTS	FORMULATION	1	2	3	µmol TEAC / 100 g	Average µmol TEAC / 100 g
ALKALIZED COCOA POWDER	A1	4291.02	4245.63	4245.63	4260.76	4134.69 ± 155.11
	B1	4169.99	4215.38	4192.68	4192.68	
	C1	3814.49	3980.89	4056.53	3950.64	
	A2	4366.66	4359.09	4351.53	4359.09	4317.07 ± 208.32
	B2	4533.06	4540.63	4487.68	4520.45	
	C2	4222.94	4018.71	3973.33	4071.66	
COCOA MASS	A1	19353.1	18884.1	18596.7	18944.61	18507.60 ± 575.342
	B1	19020.3	18702.6	18505.9	18742.91	
	C1	17568.0	17870.5	18067.2	17835.24	
	A2	16902.4	16902.4	16614.9	16806.54	16549.38 ± 251.566
	B2	16312.4	16251.9	16297.2	16287.15	
	C2	16388	16675.4	16599.8	16554.41	
DESKINNED ALMOND PASTE	A1	213.53	219.58	222.61	218.57	220.46 ± 575.33
	B1	227.14	221.85	218.07	222.35	
	A2	211.26	216.56	230.93	219.58	211.51 ± 251.55
	B2	194.62	208.99	206.72	203.44	

[1]Values represent the means ± standard deviations of 3 replicates from 3 extracts of 2 independent processing events.

It was observed that the difference between the antioxidant capacities of ingredients according to ABTS method used in the first and the second production were found to be statistically significant ($P<0.05$) (Table F.1). Additionally, total antioxidant capacities of alkalized cocoa powder, cocoa mass and deskinned almond paste according to ABTS method were compared with chocolate final products in following section 4.3.

4.2.3.2. DPPH

Total antioxidant capacities of major chocolate ingredients used in each production are presented in Table 4.5. All three formulations were evaluated together and the average total antioxidant activities of the first production were observed as 1890.52 μmol TEAC/100 g DM for alkalized cocoa powder, 12202.00 μmol TEAC/100 g DM for cocoa mass and 91.89 μmol TEAC/100 g DM for almond paste. The average total antioxidant capacities of the second production were obtained as 2406.18 μmol TEAC/100 g DM for cocoa powder, 10616.78 μmol TEAC/100 g DM for cocoa mass and 77.73 μmol TEAC/100 g DM for almond paste (Table 4.5).

Table 4.5 : Total antioxidant capacities of major chocolate ingredients used in the process according to DPPH method[1].

INGREDIENTS	FORMULATION	1	2	3	μmol TEAC / 100 g	Average μmol TEAC / 100 g
ALKALIZED COCOA POWDER	A1	1977.75	1995.87	1917.36	1963.66	1890.52 ± 123.45
	B1	1938.50	2047.20	1932.46	1972.72	
	C1	1760.35	1706.00	1739.21	1735.19	
	A2	2435.20	2395.95	2485.02	2438.72	2406.18 ± 133.86
	B2	2534.84	2543.90	2530.31	2536.35	
	C2	2293.28	2186.09	2251.01	2243.46	
COCOA MASS	A1	10326.57	11957.08	12560.98	11614.88	12202.00 ± 1161.16
	B1	14433.05	13195.06	12470.39	13366.17	
	C1	11624.94	11473.97	11775.92	11624.94	
	A2	10598.32	11005.95	10386.96	10663.75	10616.78 ± 171.27
	B2	10522.84	10492.64	10643.62	10553.03	
	C2	10673.81	10583.23	10643.62	10633.55	
DESKINNED ALMOND PASTE	A1	91.49	93.45	93.00	92.65	91.89 ± 1.90
	B1	88.47	91.49	93.45	91.14	
	A2	69.75	77.45	78.81	75.34	77.73 ± 6.07
	B2	71.86	83.94	84.55	80.12	

[1]Values represent the means ± standard deviations of 3 replicates from 3 extracts of 2 independent processing events.

It was observed that the differences between the antioxidant capacities of ingredients according to DPPH method used in the first and the second production were found to be statistically significant ($P<0.05$) (Table F.1). Total antioxidant capacities of alkalized cocoa powder, cocoa mass and deskinned almond paste according to DPPH method were compared with chocolate final products in following section 4.3.

4.2.3.3. CUPRAC

Average total antioxidant capacities of chocolate ingredients are represented in Table 4.6. All three formulations were evaluated together and the average total antioxidant activities of the first production were 5535.36 μmol TEAC/100 g DM for alkalized cocoa powder, 22494.31 μmol TEAC/100 g DM for cocoa mass and 273.91 μmol TEAC/100 g DM almond paste. The average total antioxidant activities of the second production were 6339.79 μmol TEAC/100 g DM for cocoa powder, 19813,94 μmol TEAC/100 g DM for cocoa mass and 239.03 μmol TEAC/100 g DM for almond paste (Table 4.6). It was observed that the differences between the antioxidant capacities of ingredients according to CUPRAC method used in the first and the second production were found to be statistically significant ($P<0.05$) (Table F.1).

Table 4.6 : Total antioxidant capacities of major chocolate ingredients used in the process according to CUPRAC method[1].

INGREDIENTS	FORMULATION	1	2	3	μmol TEAC / 100 g	Average μmol TEAC / 100 g
ALKALIZED COCOA POWDER	A1	5215.89	5432.71	5564.69	5404.43	5535.36 ± 175,01
	B1	5640.10	5772.08	5470.42	5627.53	
	C1	5536.41	5724.94	5460.99	5574.11	
	A2	6092.59	5970.04	6073.74	6045.46	6339.79 ± 241.62
	B2	6629.92	6507.37	6601.64	6579.65	
	C2	6441.39	6318.84	6422.53	6394.25	
COCOA MASS	A1	22627.34	22419.95	22806.45	22617.91	22494.31 ± 171.01
	B1	22636.77	22372.81	22269.12	22426.23	
	C1	22353.96	22542.50	22419.95	22438.80	
	A2	21467.83	21317.00	21448.98	21411.27	19813.94 ± 1929,92
	B2	17320.01	17197.46	17291.72	17269.73	
	C2	20826.80	20675.97	20779.67	20760.82	
DESKINNED ALMOND PASTE	A1	284.36	269.75	258.44	270.85	273.91 ± 10.38
	B1	285.78	268.81	276.35	276.98	
	A2	241.94	234.40	240.06	238.80	239.03 ± 3.48
	B2	241.94	234.87	241.00	239.27	

[1]Values represent the means ± standard deviations of 3 replicates from 3 extracts of 2 independent processing events.

Total antioxidant capacities of alkalized cocoa powder, cocoa mass and deskinned almond paste according to CUPRAC method were compared with chocolate final products in following section 4.3.

4.2.3.4. FRAP

Average total antioxidant capacities of major chocolate ingredients are represented in Table 4.7. All three formulations were evaluated together and the average total antioxidant capacities of the first production were observed as 1733.84 μmol TEAC/100 g DM for alkalized cocoa powder, 12655.41 μmol TEAC/100 g DM for cocoa mass and 86.94 μmol TEAC/100 g DM for deskinned almond paste. The average total antioxidant capacities of the second production were observed as 1986.78 μmol TEAC / 100 g DM for cocoa powder, 11157.80 μmol TEAC/100 g DM for cocoa mass and 76.04 μmol TEAC/100 g DM for almond paste (Table 4.7).

Table 4.7 : Total antioxidant capacities of major chocolate ingredients used in the process according to FRAP method[1].

INGREDIENTS	FORMULATION	1	2	3	μmol TEAC / 100 g	Average μmol TEAC / 100 g
ALKALIZED COCOA POWDER	A1	1801.32	1722.95	1747.77	1757.34	1733.84 ± 43.85
	B1	1685.08	1694.22	1670.71	1683.33	
	C1	1772.58	1764.75	1745.15	1760.83	
	A2	1977.63	1958.04	1971.10	1968.93	1986.78 ± 26.03
	B2	1978.94	1960.65	1976.33	1971.97	
	C2	2027.26	2007.67	2023.35	2019.43	
COCOA MASS	A1	13037.07	12801.98	12893.41	12910.82	12655.41 ± 393.54
	B1	12788.92	12893.41	13024.01	12902.11	
	C1	11979.17	12279.56	12201.20	12153.31	
	A2	12331.80	12135.89	12266.50	12244.73	11157.80 ± 995.38
	B2	10046.20	9837.23	10007.02	9963.48	
	C2	11352.26	11143.29	11300.02	11265.19	
DESKINNED ALMOND PASTE	A1	86.87	86.67	86.08	86.54	86.94 ± 0.82
	B1	86.93	88.50	86.60	87.34	
	A2	76.87	75.90	76.68	76.48	76.04 ± 0.38
	B2	75.96	74.98	75.83	75.59	

[1]Values represent the means ± standard deviations of 3 replicates from 3 extracts of 2 independent processing events.

It was observed that the differences between the antioxidant capacities of ingredients according to FRAP method used in the first and the second production were found to be statistically significant ($P<0.05$) (Table F.1).

Total antioxidant capacities of alkalized cocoa powder, cocoa mass and deskinned almond paste according to FRAP method were compared with chocolate final products in following section 4.3.

4.3. The Effect of Processing and Almond Content on Chocolate Phenolics

4.3.1. Total phenolic content

The average phenolic contents of three chocolate formulations in all manufacturing steps are given in Table 4.8. At first the results are presented by evaluating the changes in phenolics at each step.

The results indicated that highest total phenolic content was obtained in formulation C (726.95 mg CE/100 g dry matter (DM)), followed by formulation B (704.22 mg CE/100 g DM) and formulation A (485.84 mg CE/100 g DM) in mixing step (Table 4.8). The differences among the total phenolic contents of formulations at mixing process step were found to be significant ($P<0.05$). There was a slight decrease of about 3% for the total phenolic content of formulation B compared to the control sample, but about 33% of decrease was observed in the total phenolic content of formulation A compared to the control sample in mixing process.

Total phenolic contents of all chocolate formulations were increased significantly in refining process compared to mixing process. This increase might occur due to the reduction of particle size. It was reported that maximum reduction of particle size of sunflowers resulted higher yields in total phenolic and anthocyanin extraction (Gao and Mazza, 1996). According to the results of refining process, it was observed that highest total phenolic content was obtained in formulation C (801.56 mg CE/100 g DM), followed by formulation B (705.43 mg CE/100 g DM) and formulation A (622.48 mg CE/100 g DM) (Table 4.8). About 22% and 12% lower total phenolic contents were observed in formulation A and formulation B compared to control sample, respectively in refining process. The differences among the total phenolic contents of formulations at refining process step were found to be significant ($P<0.05$).

Table 4.8: Total phenolic contents of chocolate formulations in chocolate manufacturing steps[1].

Formulations		MIXING		REFINING		CONCHING		TEMPERING		FINAL PRODUCT	
		1	2	1	2	1	2	1	2	1	2
A	1	483.95	479.58	639.56	612.68	556.73	506.24	438.27	402.20	483.44	470.60
	2	495.53	477.83	624.05	610.93	565.03	504.05	427.99	400.45	453.52	472.58
	3	501.21	476.95	637.60	610.06	560.01	503.62	444.39	399.80	505.19	457.34
	Average	493.56	478.12	633.74	611.22	560.59	504.64	436.88	400.82	480.72	466.84
	Total Average	485.84 ± 10.16 c		622.48 ± 13.47 c		532.61 ± 30.77 c		418.85 ± 20.45 c		473.78 ± 18.83 b	
B	1	696.83	696.83	705.57	696.39	662.08	582.08	580.77	526.35	542.45	486.94
	2	713.22	693.55	730.71	693.33	659.67	579.02	569.62	522.63	538.72	503.45
	3	728.74	696.17	710.82	695.74	640.00	581.64	534.65	525.47	507.08	502.18
	Average	712.93	695.52	715.70	695.15	653.92	580.92	561.68	524.82	529.42	497.52
	Total Average	704.22 ± 13.93 b		705.43 ± 14.07 b		617.42 ± 40.72 b		543.25 ± 25.31 b		513.47 ± 22.14 a	
C	1	726.12	714.97	824.69	783.38	733.11	765.90	601.97	626.01	500.60	479.09
	2	756.93	709.72	812.23	777.48	732.24	759.99	576.62	619.89	482.44	474.03
	3	740.10	713.88	830.15	781.41	749.50	763.93	613.77	623.61	492.55	475.22
	Average	741.05	712.86	822.36	780.76	738.28	763.27	597.45	623.17	491.86	476.11
	Total Average	726.95 ± 18.35 a		801.56 ± 23.59 a		750.78 ± 15.13 a		610,31 ± 18.61 a		483.99 ± 10.50 b	

[1]Values represent the means ± standard deviations of 3 replicates of 2 independent processing events. All contents are expressed as mg CE/100 g dry weight. Different letters in the columns represent statistically significant differences between formulations ($P<0.05$).

Total phenolic contents of all chocolate formulations were decreased at conching step according to thermal treatments (70°C). The results showed that highest total phenolic content was obtained in formulation C (750.78 mg CE/100 g DM), followed by formulation B (617.42 mg CE/100 g DM) and formulation A (532.61 mg CE/100 g DM) (Table 4.8). According to the comparison of the total phenolic contents of formulation A and formulation B to the control sample, about 29% and 18% lower values were observed, respectively. The differences among the total phenolic contents of formulations at conching process step were found to be significant ($P<0.05$).

Total phenolic contents of chocolate formulations decreased in following tempering process. This decrease might occur due to continuous heating (46°C) and cooling (32°C) of the chocolate. The results revealed that highest total phenolic content was obtained in formulation C (610.31 mg CE/100 g DM), followed by formulation B (543.25 mg CE/100 g DM) and formulation A (418.85 mg CE/100 g DM) (Table 4.8).

In tempering process, the total phenolic contents of formulation A and formulation B found to be about 31% and 11% lower than the control sample, respectively. The differences among the total phenolic contents of formulations at tempering process step were found to be significant ($P<0.05$).

According to the results of final products, formulation C had a total phenolic content of 483.99 mg CE/100 g DM, whereas formulation B and A had total phenolic contents of 513.47 mg CE/100 g DM and 473.78 mg CE/100 g DM, respectively (Table 4.8). The differences among the total phenolic contents of chocolate final products were found to be significant ($P<0.05$).

Additionally, total phenolic contents of alkalized cocoa powder, cocoa mass and deskinned almond paste were compared with chocolate final products. Total phenolic content of alkalized cocoa powder ranged from 475.91 mg CE/100 g DM to 544.76 mg CE/100 g DM (Table 4.2). In comparison with chocolate final products, total phenolic content of alkalized cocoa powder was found to be slightly higher than chocolate final products. Total phenolic content of cocoa mass ranged from 1650.26 mg CE/100 g DM to 1865.91 mg CE/100 g DM (Table 4.2). These values were found to be higher than 3-fold of the values obtained from chocolate final products.

Total phenolic content of deskinned almond paste ranged from 8.59 mg CE/100 g DM to 10.42 mg CE/100 g DM (Table 4.2) and these values were very low compared to other cocoa product extracts. The decreasing order of the extracts based on their total phenolic contents: cocoa mass > alkalized cocoa powder > final products >> deskinned almond paste.

According to the literature data, total phenolic contents of cocoa and chocolate extracts vary greatly depending on the solvent and procedure used for the extraction of polyphenols. It has also been known that depending on the cocoa bean variety, geographical origin, ripeness degree (harvest season) and post-harvest conditions, such as fermentation, drying, roasting, processing and storage (Wollgast and Anklam, 2000). Using the Folin-Ciocalteu's method, Waterhouse et al (1996) have found 8.4 mg polyphenols/g dark chocolate and 5.0 mg polyphenols/g milk chocolate given as gallic acid equivalents. Vinson et al. (1999) found higher values (36.5 mg/g dry matter for dark chocolate and 15 mg/g dry matter for milk chocolate using the same method but catechin as a standard. These values are high compared to the results obtained by Adamson et al. (1999) using high performance liquid chromatograph for the quantification of catechins and procyanidins, milk chocolate having 0.7 mg polyphenols/g and dark chocolate having 1.7 mg polyphenols/g. Arts et al. (1999) measured the content of catechins only and found dark chocolate having 0.5 mg/g and milk chocolate having about 0.2 mg/g, respectively and the results of our study are in agreement with these values.

Andres-Lacueva et al (2008) studied alkalized cocoa powder derived products and produced product B with 40-42% alkalized cocoa powder and product C with 16-18% alkalized cocoa powder and measured the total phenolic content. The total phenolic content of product B was 530,41 μg (-)-epicatechin equivalents (ECE)/g DM whereas product C was 223.47 μg ECE/g DM. The results of this study cannot be compared to these values because of using different standards and extraction solvents.

In general, the samples of formulation A, formulation B and formulation C were found to be changed similarly in all manufacturing steps. Generally, the total phenolic content of the control sample showed the highest value, followed by formulation B and formulation A.

The results of our study suggested that the almond addition to chocolate decrease the total phenolic content. It might occur according to the polyphenol-protein interactions. A large number of *in vitro* studies have suggested that these interactions involve both the hydrogen bonds and hydrophobic interactions between hydroxyl groups of the phenolic compounds and carbonyl groups of proteins (Spencer et al.,1988). These interactions might lead to masking of phenolic compounds.

Almonds are often used in chocolate production. They are rich source of vitamin E so that several manufacturers produce dark chocolate products with almonds that contain phenolic compounds. Amandin is the primary storage protein in almonds. Tiwari et al. (2010) who investigated amandin recovery from chocolate matrix in general and dark chocolate presented interesting results. Their results suggested that dark chocolate significantly decreased amandin recovery when dark chocolate was spiked with amandin prior to protein extraction (Tiwari et al., 2010). A similar interaction between the phenolic compounds and proteins has also been reported by Sharma et al. (2008) in black tea, added with sugar, milk or both. They reported that black tea extracts had a maximum amount of polyphenol content followed by black tea with sugar and black tea with milk and sugar. In addition, total polyphenol contents were found to be the lowest compared to other samples in black tea with milk. Those decreases were explained by either covalently and non-covalently interactions between plant phenolics and proteins. Both covalent and non-covalent interactions might lead to precipitation of proteins through multisite interactions and multidentate interactions. In multisite interactions, several phenolics bind to one protein molecule and in multidentate interactions one phenolic bind to several protein sites or protein molecules. These interactions might lead to masking of polyphenol content such as catechins and flavonoids on the proteins (Arts et al., 2002).

The reduction of phenolics absorption in the presence of milk is in agreement with Serafini et al. (2003). They reported that after consumption of chocolate with milk or milk chocolate the peak concentrations of plasma flavan-3-ols were found to be decreased (Serafini et al., 2003).

Komes et al. (2009) studied the total phenolic contents of various chocolate products. They reported that the lowest total phenolic content was observed in milk chocolate although it contains higher cocoa solids content (29%) than cocoa bars (16%). It was suggested that this decrease resulted from strong catechin-protein interactions. Milk

based products represent a very complex matrix that strong catechin-protein interactions are well-known to occur and it directly influences catechin determination by significantly reducing analytical recovery from the food matrix (Komes et al., 2009).

As a comparison of chocolate manufacturing process steps in all formulations it was observed that the highest phenolic contents were obtained from refining steps, followed by mixing, conching and tempering steps. Figure 4.1 represents the comparison of total phenolic contents of all manufacturing steps of chocolate samples.

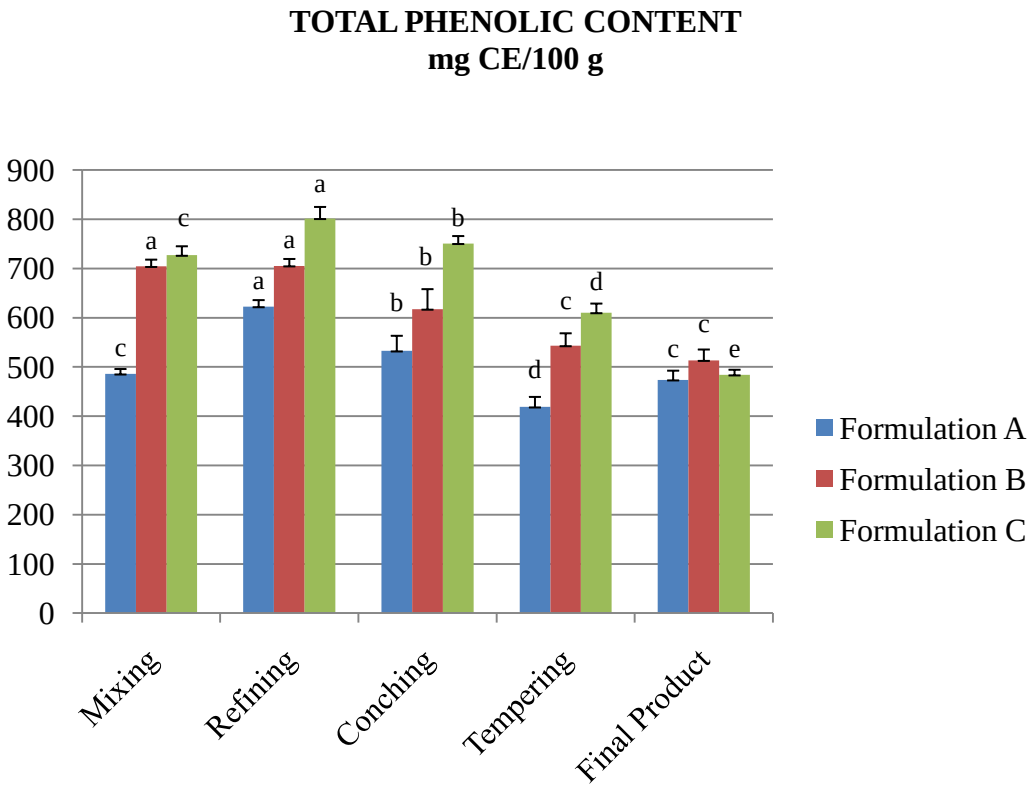

Figure 4.1 : Comparison of total phenolic contents of all manufacturing steps of chocolate samples[1].

[1]Different letters on the columns represent statistically significant differences ($p<0.05$).

Total phenolic content of formulation A increased about 28% in refining step compared to mixing step. It was decreased about 14% and 21% in following conching and tempering steps, respectively. Although total phenolic content of refining step increased slightly in formulation B, about 12% and 12% reductions were observed in following conching and tempering steps, respectively. In

formulation C, total phenolic content increased by 10% in refining process compared to mixing process. It was later decreased by 6% and 18% in conching and tempering processes compared to prior steps. The difference of total phenolic contents of each manufacturing process steps of all chocolate formulations were found to be significant ($P<0.05$).

4.3.2. Total flavonoid content

The average phenolic contents of three chocolate formulations in all manufacturing steps are given in Table 4.9. At first, the results are presented by evaluating the changes in total flavonoids at each step.

The results indicated that highest total flavonoid content was obtained in formulation C (573.81 mg CE/100 g DM), followed by formulation B (519.42 mg CE/100 g DM) and formulation A (437.85 mg CE/100 g DM) in mixing step (Table 4.9). The differences among the total flavonoid contents of formulations at mixing process step were found to be significant ($P<0.05$).

About 24% and 10% lower total flavonoid contents were observed in formulation A and formulation B compared to control sample in mixing process, respectively.

According to the results of refining process, it was observed that highest total flavonoid content was obtained in formulation C (759.65 mg CE/100 g DM), followed by formulation B (594.26 mg CE/100 g DM) and formulation A (560.35 mg CE/100 g DM) (Table 4.9). The differences among the total flavonoid contents of formulations at refining process step were found to be significant ($P<0.05$).

According to the comparison of the total flavonoid contents of formulation A and formulation B to the control sample in refining process, about 26% and 22% lower values were observed, respectively.

The results obtained from conching step demonstrated that highest total flavonoid content was obtained in formulation C (567.28 mg CE/100 g DM), followed by formulation B (487.56 mg CE/100 g DM) and formulation A (394.61 mg CE/100 g DM) (Table 4.9). In conching process, formulation A and formulation B gave lower results of 30% and 14% compared to the control sample, respectively. The differences among the total flavonoid contents of formulations at conching process step were found to be significant ($P<0.05$).

Table 4.9 : Total flavonoid contents of chocolate formulations in chocolate manufacturing steps[1].

Formulations		MIXING		REFINING		CONCHING		TEMPERING		FINAL PRODUCT	
		1	2	1	2	1	2	1	2	1	2
A	1	444.26	432.72	568.39	552.65	407.90	376.43	379.93	326.43	382.69	386.61
	2	445.31	427.48	570.49	547.41	400.91	370.49	346.36	321.19	365.59	375.42
	3	444.96	432.37	571.53	551.60	436.92	375.03	333.77	325.38	411.57	357.70
	Average	444.85	430.86	570.14	550.56	415.24	373.98	353.35	324.33	386.61	367.57
	Total Average	437.85 ± 7.89 c		560.35 ± 10.91 c		394.61 ± 25.70 c		338.84 ± 21.99 c		377.09 ± 18.91 b	
B	1	525.13	521.90	603.70	587.97	515.94	460.35	403.35	390.77	425.85	396.31
	2	516.56	516.45	601.95	582.02	544.96	455.80	431.33	386.22	421.78	400.77
	3	520.67	515.78	603.35	586.57	488.32	460.00	413.49	390.42	395.39	401.41
	Average	520.79	518.04	603.00	585.52	516.41	458.71	416.06	389.13	414.34	399.50
	Total Average	519.07 ± 3.76 b		594.26 ± 9.79 b		487.56 ± 36.36 b		402.60 ± 17.33 b		406.92 ± 13.36 a	
C	1	569.09	564.89	769.44	742.16	599.86	579.23	429.58	436.92	390.30	379.07
	2	581.32	559.30	793.91	736.22	527.48	573.63	395.66	431.33	382.89	375.22
	3	604.05	564.19	775.38	740.76	546.01	577.48	426.08	435.87	403.80	374.16
	Average	584.82	562.79	779.57	739.72	557.78	576.78	417.11	434.71	392.33	376.15
	Total Average	573.81 ± 16.59 a		759.65 ± 23.36 a		567.28 ± 26.02 a		425.91 ± 15.35 a		384.24 ± 11.23 b	

[1]Values represent the means ± standard deviations of 3 replicates from 3 extracts of 2 independent processing events. All contents are expressed as mg CE/100 g dry weight. Different letters in the coloumns represent statistically significant differences between formulations (P<0.05).

In tempering step, the results showed that highest total flavonoid content was obtained in formulation C (425.91 mg CE/100 g DM), followed by formulation B (402.60 mg CE/100 g DM) and formulation A (338.84 mg CE/100 g DM) (Table 4.9). The differences among the total flavonoid contents of formulations at tempering process step were found to be significant ($P<0.05$).

According to the results obtained from the tempering process, slightly lower values were observed for formulation B (5%), but the difference between the total flavonoid contents of formulation A and control sample was much higher (20%).

According to the results of final products, formulation C had a total flavonoid content of 384,24 mg CE/100 g DM, whereas formulation B and A had total flavonoid contents of 406,92 mg CE/100 g DM and 377,09 mg CE/100 g DM, respectively (Table 4.9). The differences among the total flavonoid contents of chocolate final products were found to be significant ($P<0.05$).

In addition, total flavonoid contents of alkalized cocoa powder, cocoa mass and deskinned almond paste were compared with chocolate final products. Total flavonoid content of alkalized cocoa powder ranged from 304.17 mg CE/100 g DM to 348.46 mg CE/100 g DM (Table 4.3). In comparison with chocolate final products, total flavonoid content of chocolate final products was slightly higher than alkalized cocoa powder. Total flavonoid content of cocoa mass ranged from 1713.38 mg CE/100 g DM to 1511.05 mg CE/100 g DM (Table 4.3). These values were found to be higher than the values obtained from chocolate final products. Total flavonoid content of deskinned almond paste ranged from 6.61 mg CE/100 g DM to 7.57 mg CE/100 g DM (Table 4.3) and these values were very low compared to other cocoa product extracts. The decreasing order of the extracts based on the total flavonoid contents: cocoa mass > final products > alkalized cocoa powder >> deskinned almond paste.

Generally, in all the process steps the total flavonoid content values gave the smilar trend for all chocolate formulations. Similar to the order of total phenolic content, the total flavonoid content of control samples gave the highest values for formulation C, followed by formulation B and A.

It was suggested that almond addition to chocolate formulations decrease total flavonoid content due to a possible flavonoid-protein binding in this study. Komes et

al. (2009) studied total flavonoid contents of various chocolate products. They reported that the lowest total flavonoid contents were observed in milk chocolate although it contains higher cocoa solids content (29%) than cocoa bars (16%).

Pimentel et al. (2009) reported the total flavonoid content of dark chocolate with 71% cocoa and dark chocolate with 40% cocoa as 21.6 ± 2.4 and 17.2 ± 1.3 μmol of catechin equivalents/g, respectively. The results presented in this study are in agreement with this data when compared to μmol of catechin equivalents/g for formulation C which was 13.24 μmol of catechin equivalents/g. However, the results cannot be directly compared due to the differences in the applied extraction solvent. Additionally, some literature data were expressed as mg of standard compound per g of cocoa product, while our research data were given as mg of standard compound per gram of defatted cocoa, which also contributes to significant differences between the results.

All total flavonoid contents of chocolate formulations were compared to evaluate the effect of processing on chocolate and it was observed that the highest flavonoid contents were obtained from refining steps, followed by mixing, conching and tempering steps. Figure 4.2 shows the average total flavonoid contents of all manufacturing process steps of all formulations.

Total flavonoid contents were increased about 28%, 14% and 32% in refining steps of formulations A, B and C, compared to the mixing step, respectively. In conching step, total flavonoid contents of formulation A, B and C were lower than the refining steps about 29%, 18% and 25%, respectively. Later in tempering steps, total flavonoid contents continued decreasing due to rapid heating (46°C) and cooling (32°C) of chocolate samples and indicated 14%, 18% and 25% lower values for formulations A, B and C compared to the conching step, respectively. Total flavonoid contents of each manufacturing process steps of all chocolate formulations were found to be significant ($P<0.05$).

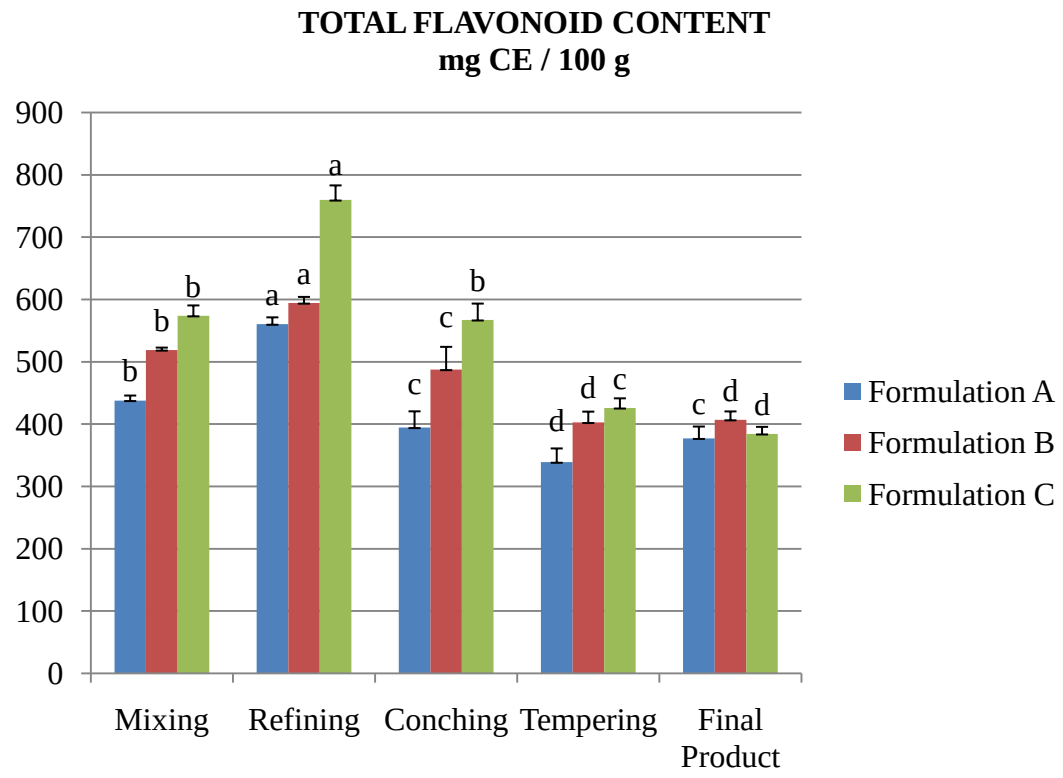

Figure 4.2 : Comparison of total flavonoid contents of all manufacturing steps of chocolate samples[1].

[1]Different letters on the columns represent statistically significant differences ($p<0.05$).

4.3.3. Total antioxidant activity

There are many methods used in the literature to determine the antioxidant capacities of cocoa and cocoa products. Single antioxidant capacity measurement method cannot examine all the antioxidant compounds as all methods have their own advantages or disadvantages (Capanoglu et al., 2008). These methods vary greatly according to the generated radicals, the reaction time and end-point of detection. Although ABTS and DPPH methods work with the same principle, the results vary according to their response to antioxidants under certain conditions. There are also variability in radical formation and solubility in different solvent systems (Arnao, 2000; Apak et al., 2007). Results of the antioxidant activity measurement methods cannot be evaluated satisfactorily using a single method because of the great variability influencing the results. In order to evaluate the antioxidant activity correctly, using several test methods for measuring antioxidant capacities is highly recommended (Capanoglu et al., 2010).

4.3.3.1. ABTS

The total antioxidant capacities of chocolate formulations from all process steps are represented in Table 4.10. The results indicated that highest total antioxidant capacity was obtained in formulation C (6637.10 mg CE/100 g DM), followed by formulation B (5774.70 mg CE/100 g DM) and formulation A (5275.22 mg CE/100 g DM) in mixing step (Table 4.10). About 21% and 13% lower antioxidant activity values were obtained, respectively for formulation A and formulation B compared to control sample in mixing step. The differences among the total antioxidant capacities of formulations at mixing process step were found to be significant ($P<0.05$).

Results of refining step showed that highest total antioxidant activity was obtained in formulation C (7817.07 μmol TEAC/100 g DM), followed by formulation B (7504.43 μmol TEAC/100 g DM) and formulation A (6275.29 μmol TEAC/100 g DM) (Table 4.10). The differences among the total antioxidant capacities of formulations at refining process step were found to be significant ($P<0.05$). In refining process, about 20% and 4% lower antioxidant capacities were detected for formulation A and formulation B as a result of comparison with control sample.

According to the results of refining process, it was observed that the total phenolic content, total flavonoid content and the total antioxidant capacity were significantly increased ($P<0.05$) compared to the mixing step. The particle size might be a factor resulting an increase in antioxidant capacity as similar increases have been reported in various food commodities such as blackcurrant juice press residues (Landbo and Meyer, 2001), black cohosh (Mukhopadhyay et al., 2006) and wheat (Cheng et al., 2006).

It is suggested that this *in vitro* antioxidant capacity increase resulted according to the reduction of particle size. Reduction of particle size breaks up certain structures of the food matrix and releases bound antioxidants and thereby reducing the distance the analyte has to travel to the surface. Moreover, it is known that particle surface enlargement may improve solvent penetration, which in turn increase the efficiency of extraction (Perezjimenez et al., 2008).

Table 4.10 : Total antioxidant capacities of chocolate formulations in chocolate manufacturing steps according to ABTS method[1].

Formulations		MIXING		REFINING		CONCHING		TEMPERING		FINAL PRODUCT	
		1	2	1	2	1	2	1	2	1	2
A	1	5024.72	5305.69	6318.15	6212.25	4631.39	4064.10	3791.80	3996.02	5113.22	4756.01
	2	5402.91	5238.78	6401.35	6280.33	4707.03	4856.04	3935.51	3844.74	4840.49	4780.16
	3	5425.60	5253.70	6408.92	6030.72	4941.51	4117.05	4055.13	3602.70	5407.58	4685.19
	Average	5284.41	5266.04	6376.14	6174.43	4759.98	4345.73	3927.48	3814.49	5120.43	4740.45
	Total Average	5275.22 ± 144.49 c		6275.29 ± 141.00 c		4552.85 ± 374.63 c		3870.99 ± 162.88 c		4930.44 ± 276.52 b	
B	1	5713.04	5874.15	7770.42	7157.75	5848.98	5169.80	4850.75	4464.99	5478.61	5333.92
	2	5733.12	5632.10	7528.38	7422.48	5553.36	4986.02	4874.15	4502.81	5478.61	5308.36
	3	5962.64	5733.12	7566.20	7581.32	5561.33	5121.85	4608.70	4298.58	5124.74	5137.28
	Average	5802.93	5746.46	7621.67	7387.18	5647.89	5092.56	4777.87	4422.12	5349.74	5259.86
	Total Average	5774.69 ± 120.67 a		7504.43 ± 203.94 b		5370.22 ± 329.19 b		4600.00 ± 226.56 b		5304.80 ± 149.30 a	
C	1	6711.95	6721.31	8541.94	7513.25	6060.73	6348.38	5274.33	5387.79	5058.96	5076.81
	2	6915.70	6751.90	7921.70	7717.48	5653.22	6332.40	5009.59	5372.66	5130.51	4952.63
	3	6696.35	6025.42	7581.33	7626.71	5393.53	6312.43	5138.18	5281.89	5348.45	4886.09
	Average	6774.66	6499.54	8014.99	7619.15	5702.49	6331.07	5140.70	5347.45	5179.31	4971.84
	Total Average	6637.09 ± 310.21 a		7817.07 ± 382.21 a		6016.78 ± 404.85 a		5244.07 ± 145.42 a		5075.57 ± 160.48 ab	

[1]Values represent the means ± standard deviations of 3 replicates from 3 extracts of 2 independent processing events. All contents are expressed as µmol TEAC/100 g dry weight. Different letters in the columns represent statistically significant differences between formulations (P<0.05).

Although particle size reduction increases *in vitro* antioxidant capacity, it is important to note that it may also reduce the thermal stability (Cheng et al., 2006; Perezjimenez et al., 2008). Therefore, the decrease of the antioxidant capacities in the following conching and tempering steps might be resulted from degradation of some antioxidant compounds according to the reduced thermal stability (70°C at conching steps and 32-46°C at tempering steps).

In conching process, the highest total antioxidant activity was obtained in formulation C (6016.78 μmol TEAC/100 g DM), followed by formulation B (5370.22 μmol TEAC/100 g DM) and formulation A (4552.85 μmol TEAC/100 g DM) (Table 4.10). As a result of these findings it was considered that 24% and 11% lower antioxidant capacities were obtained, respectively for formulation A and formulation B compared to the control sample in conching process. The differences among the total antioxidant capacities of formulations at conching process step were found to be significant ($P<0.05$).

The results revealed that highest total antioxidant activity was obtained in formulation C (5244.07 μmol TEAC/100 g DM), followed by formulation B (4600.00 μmol TEAC/100 g DM) and formulation A (3870.99 μmol TEAC/100 g DM) (Table 4.10). The differences among the total antioxidant activities of formulations at tempering process step were found to be significant ($P<0.05$).

In tempering process, about 26% and 12% lower values for antioxidant capacities of formulation A and formulation B were obtained, respectively as a comparison with the control sample.

According to the results of final products, formulation C had an antioxidant activity of 5075.57 μmol TEAC/100 g DM, whereas formulation B and A had activity values of 5304.80 μmol TEAC/100 g DM and 4930.43 μmol TEAC/100 g DM, respectively (Table 4.10). The differences among the total antioxidant capacities of chocolate final products were found to be significant ($P<0.05$).

Additionally, total antioxidant capacities of alkalized cocoa powder, cocoa mass and deskinned almond paste according to ABTS method were compared with chocolate final products. Total antioxidant capacity of alkalized cocoa powder ranged from 4134.69 μmol TEAC/100 g DM to 4317.07 μmol TEAC/100 g DM (Table 4.4). In comparison with chocolate final products total antioxidant capacity of chocolate last

products were found to be higher than alkalized cocoa powder. Total antioxidant capacity of cocoa mass ranged from 16549.38 μmol TEAC/100 g DM to 18507.60 μmol TEAC/100 g DM (Table 4.4). These values were found to be higher compared to chocolate final products. Total antioxidant capacity of deskinned almond paste was very low compared to other cocoa product extracts. The decreasing order of the extracts based on the total antioxidant capacities observed with ABTS assay: cocoa mass > final products > alkalized cocoa powder >> deskinned almond paste.

Comparing the results of the applied ABTS assay to measure antioxidant capacity, it can be noticed that almond addition decrease the total antioxidant capacity as a result of flavonoid-protein binding. The study of Komes et al. (2009) completely in agreement with our findings that according to ABTS method, lowest total antioxidant capacity were observed in milk chocolate although it contains higher cocoa solids content (29%) than cocoa bars (16%) as a result of catechin-protein interactions.

The comparison between studies of antioxidant capacity measurements reported from different laboratories is quite difficult because of substantial differences in sample preparation, extraction methods of antioxidants and expression of the results (Perezjimenez et al., 2008). Komes et al. (2009) studied antioxidant capacity of dark chocolates with 88% cocoa solids, 72% cocoa solids and 60% cocoa solids and reported antioxidant capacity of 2040 μmol TEAC/100 g DM, 1773 μmol TEAC/100 g DM and 1801 μmol TEAC/100 g DM using ABTS method, respectively. These values are lower than the values observed from the chocolate products analyzed in this study. This difference may depend on the cocoa bean variety used in the chocolate production, chocolate manufacturing conditions and storage. Besides extraction method may also affect this difference. In the study of Komes et al. (2009) 70% methanol was used for extraction. However, our preliminary results (Section 4.1) on cocoa beans, it was observed that 80% methanol extraction yielded significantly higher values ($P<0.05$) than extraction with 70% methanol.

All total antioxidant capacity values of chocolate formulations according to ABTS method were compared to evaluate the effect of processing on chocolate and it was observed that the highest total antioxidant capacity values were obtained from refining steps, followed by mixing, conching and tempering steps. Figure 4.3 shows the average total antioxidant activities of all manufacturing process steps of all formulations.

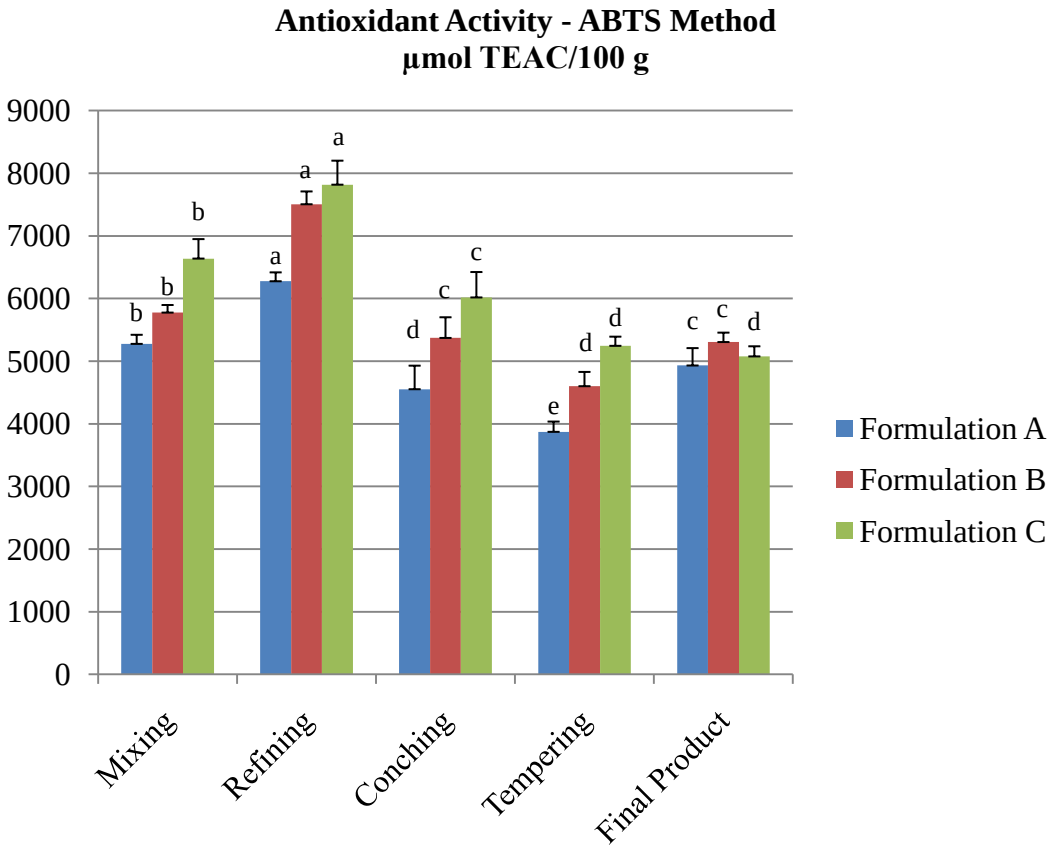

Figure 4.3 : Comparison of total antioxidant capacities of all manufacturing steps of chocolate samples according to ABTS method[1].

[1]Different letters on the columns represent statistically significant differences ($p<0.05$).

The antioxidant capacities of refining process samples increased about 19%, 30%, and 18% for formulations A, B and C, compared to prior mixing step, respectively. After the refining step, total antioxidant capacities decreased in following conching and tempering steps according to thermal treatments (70°C at conching step and 32-46°C at tempering step). Refining step decreased the thermal stability of antioxidant compounds due to increased surface area of particles. In conching step, the antioxidant capacities of formulation A, B and C were found to be about 27%, 28% and 23% lower than prior refining step. Then, antioxidant capacities continued decreasing in tempering step and 15%, 14% and 13% lower values were observed compared to conching step values for formulation A, B and C, respectively. Total antioxidant activities of each manufacturing process steps of all chocolate formulations were significant ($P<0.05$).

4.3.3.2. DPPH

The total antioxidant activities of chocolate formulations from all process steps are represented in Table 4.11. The results illustrated that highest total antioxidant activity was obtained in formulation C (4493.97 μmol TEAC/100 g DM), followed by formulation B (4153.78μmol TEAC/100 g DM) and formulation A (3283.67 μmol TEAC/100 g DM) in mixing process (Table 4.11). The differences among the total antioxidant activities of formulations at mixing process step were found to be significant ($P<0.05$). In mixing process, antioxidant capacities of formulation A and B were found to be lower (27% and 8%, respectively) compared to the control samples.

According to the results of refining process, it was observed that highest total antioxidant activity was obtained in formulation C (4931.29 μmol TEAC/100 g DM), followed by formulation B (4197.56 μmol TEAC/100 g DM) and formulation A (3765.78 μmol TEAC/100 g DM) (Table 4.11). The differences among the total antioxidant activities of formulations at refining process step were found to be significant ($P<0.05$). As a comparison of the formulation A and formulation B antioxidant capacities to the control samples, 24% and 15% lower values were obtained, respectively.

The results obtained from conching step indicated that highest total antioxidant activity was obtained in formulation C (4331.42 μmol TEAC/100 g DM), followed by formulation B (3675.19 μmol TEAC/100 g DM) and formulation A (2801.56 μmol TEAC/100 g DM) (Table 4.11). The differences among the total antioxidant activities of formulations at conching process step were found to be significant ($P<0.05$). The results indicated that the antioxidant capacities of formulation A and formulation B found to be lower than the control samples (35% and 15%, respectively).

The results of the tempering process illustrated that highest total antioxidant activity was obtained in formulation C (3686.26 μmol TEAC/100 g DM), followed by formulation B (3322.42 μmol TEAC/100 g DM) and formulation A (2506.66μmol TEAC/100 g DM) (Table 4.11).

Table 4.11 : Total antioxidant capacities of chocolate formulations in chocolate manufacturing steps according to DPPH method[1].

Formulations		MIXING		REFINING		CONCHING		TEMPERING		FINAL PRODUCT	
		1	2	1	2	1	2	1	2	1	2
A	1	3478.42	3288.20	3816.60	3668.65	2904.73	2660.15	2687.32	2406.51	3094.25	3011.43
	2	3248.94	3261.02	3834.72	3704.88	3031.54	2620.90	2654.11	2352.16	2873.94	2967.92
	3	3264.04	3276.07	3858.88	3710.92	2956.06	2635.99	2551.45	2388.40	3243.29	2956.42
	Average	3330.47	3236.87	3836.73	3694.82	2964.11	2639.01	2630.96	2382.36	3070.49	2978.59
	Total Average	3302.78 ± 104.89 c		3765.78 ± 80.19 c		2801.56 ± 183.00 c		2506.66 ± 144.41 c		3024.54 ± 129.16 b	
B	1	4194.04	4043.06	4345.01	4136.67	3876.99	3505.60	3418.03	3297.26	3535.91	3170.21
	2	4290.66	4106.47	4191.02	4145.73	3858.88	3478.42	3433.13	3324.43	3410.68	3161.58
	3	4218.19	4070.24	4224.23	4142.71	3840.76	3490.50	3094.95	3366.70	3240.56	3217.47
	Average	4234.30	4073.26	4253.42	4141.70	3858.88	3491.51	3315.37	3329.46	3362.38	3183.09
	Total Average	4153.78 ± 95.90 b		4197.56 ± 79.87 b		3675.19 ± 201.73 b		3322.42 ± 123.08 b		3272.74 ± 120.51 a	
C	1	4619.78	4411.44	5066.66	4794.91	4293.68	4384.26	3602.22	3732.06	3176.68	3021.51
	2	4523.16	4378.22	5045.53	4794.91	4296.70	4411.44	3662.61	3759.23	3095.80	2967.10
	3	4640.92	4390.30	5078.74	4806.99	4197.06	4405.40	3547.87	3813.58	3174.12	3056.96
	Average	4594.62	4393.32	5063.64	4798.94	4262.48	4400.37	3604.24	3768.29	3093.95	3015.19
	Total Average	4493.97 ± 117.67 a		4931.29 ± 145.44 a		4331.42 ± 84.09 a		3686.26 ± 100.41 a		3082.03 ± 83.82 b	

[1]Values represent the means ± standard deviations of 3 replicates from 3 extracts of 2 independent processing events. All contents are expressed as μmol TEAC/100 g dry weight. Different letters in the columns represent statistically significant differences between formulations ($P<0.05$).

Similarly to the conching process, antioxidant capacities of formulation A and formulation B were 32% and 10% lower than the control sample in tempering process, respectively. The differences among the total antioxidant capacities of formulations at tempering process step were found to be significant ($P<0.05$).

According to the results of final products, formulation C had an antioxidant activity of 3082.03 μmol TEAC/100 g DM, whereas formulation B and A had activity values of 3272.74 μmol TEAC/100 g DM and 3024.54 μmol TEAC/100 g DM, respectively (Table 4.11). The differences among the total antioxidant capacities of chocolate final products were found to be significant ($P<0.05$).

Additionally, total antioxidant capacities of alkalized cocoa powder, cocoa mass and deskinned almond paste according to DPPH method were compared with chocolate final products. Total antioxidant capacity of alkalized cocoa powder ranged from 1890.52 μmol TEAC/100 g DM to 2406.18 μmol TEAC/100 g DM (Table 4.5). In comparison with chocolate final products total antioxidant capacities of chocolate final products were found to be higher than alkalized cocoa powder. Total antioxidant capacity of cocoa mass ranged from 12202.0 μmol TEAC/100 g DM to 10616.78 μmol TEAC/100 g DM (Table 4.5). These values were found to be higher compared to chocolate final products. Total antioxidant capacity of deskinned almond paste was very low compared to other cocoa product extracts. The decreasing order of the extracts based on the total antioxidant capacity observed with DPPH assay: cocoa mass > final products > alkalized cocoa powder >> deskinned almond paste.

Comparing the results of the applied DPPH assay to measure antioxidant capacity, it can be noticed that almond addition decrease the total antioxidant capacity as a result of flavonoid-protein binding. The study of Sharma et al. (2008) is completely in agreement with our findings as they compared the antioxidant capacities of four types of black tea brews: Black tea, black tea with sugar, black tea with milk, black tea with sugar and milk using the DPPH method. The antioxidant capacity of black tea found to be the highest, followed by black tea with sugar and black tea with milk and sugar. The antioxidant capacity of black tea with milk was found to be lesser compared to all the black tea extracts in all the cases. This was because of the proteins reducing the radical scavenging capacity and that the radical scavengers do

not reach their optimum scavenging capacity according to the presence of proteins (Arts et al., 2002).

Komes et al. (2009) also reported the antioxidant capacities of chocolate samples with 88%, 72% and 60% cocoa solids, 1175 μmol TEAC/100 g DM, 1162 μmol TEAC/100 g and 1147 μmol TEAC/100 g DM, respectively. As a result of comparison with the results of this study, it was observed that these values are found to be low. This difference may be a result of using different varieties of cocoa in chocolate processing, chocolate processing conditions and storage conditions. Moreover, exraction solvents used are different. Komes et al. (2009) used 70% methanol extraction in their study and it gives lower values when compared to 80% methanol extraction.

All total antioxidant capacity values of chocolate formulations according to DPPH method were compared to evaluate the effect of processing on chocolate and it was observed that the highest total antioxidant capacity values were obtained from refining steps, followed by mixing, conching and tempering steps. Figure 4.4 shows the average total antioxidant activities of all manufacturing process steps of all formulations according to DPPH method.

Antioxidant capacities of formulation A, B and C were found to be about 11%, 1% and 10% higher than mixing process antioxidant capacity values, respectively. In conching process lower antioxidant activity values (25%, 12% and 12%) were observed for formulations A,B and C, respectively. In tempering process, antioxidant capacity values continued decreasing and 10%, 10% and 15% lower values than conching step values were observed for formulations A, B and C, respectively. Total antioxidant activities of each manufacturing process steps of all chocolate formulations were significant ($P<0.05$).

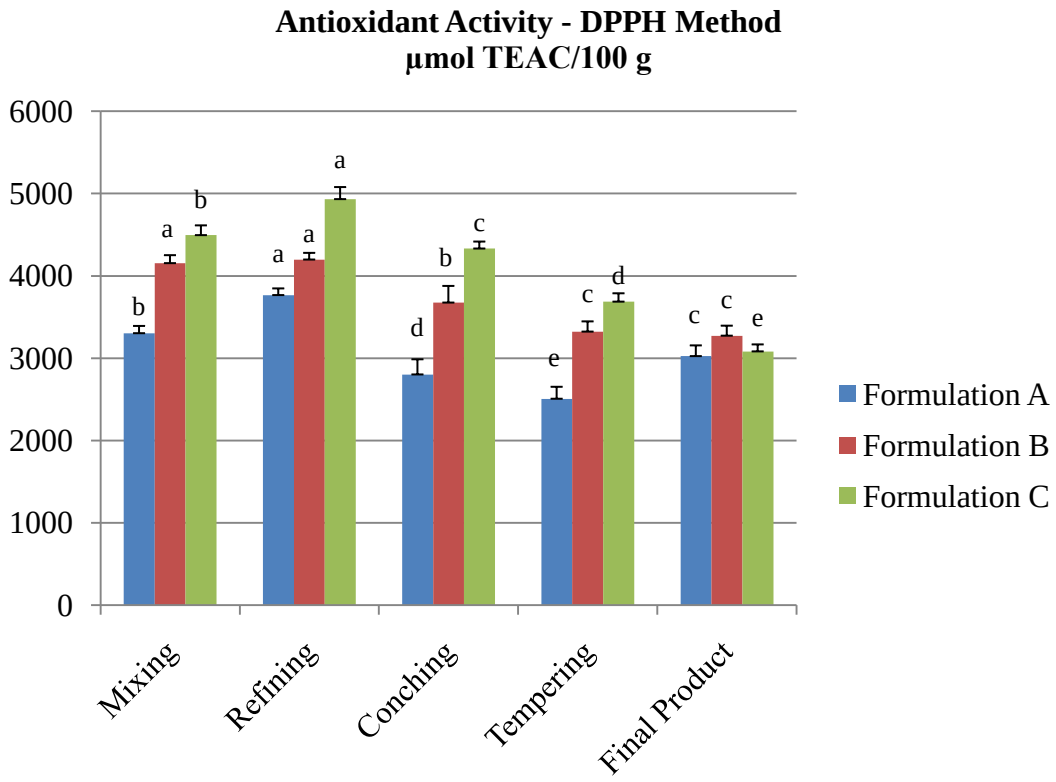

Figure 4.4 : Comparison of total antioxidant capacities of all manufacturing steps of chocolate samples according to DPPH method[1].

[1]Different letters on the columns represent statistically significant differences ($p<0.05$).

4.3.3.3. CUPRAC

Table 4.12 and Figure 4.5 represent the total antioxidant capacities of three chocolate formulations in all process steps. The results indicated that highest total antioxidant activity was obtained in formulation C (9836.63 µmol TEAC/100 g DM), followed by formulation B (7852.28 µmol TEAC/100 g DM) and formulation A (6798.04 µmol TEAC/100 g DM) in mixing step (Table 4.12).

The differences among the total antioxidant capacities of formulations at mixing process step were found to be significant ($P<0.05$). According to these antioxidant capacity values, formulation A and formulation B had about 31% and 20% lower antioxidant capacities compared to control samples in mixing process.

According to the results of refining process, it was observed that highest total antioxidant activity was obtained in formulation C (10395.96 µmol TEAC/100 g DM), followed by formulation B (9514.55 µmol TEAC/100 g DM) and formulation A (8694.41 µmol TEAC/100 g DM) (Table 4.12). The differences among the formulations at refining process were found to be significant ($P<0.05$).

Table 4.12 : Total antioxidant capacities of chocolate formulations in chocolate manufacturing steps according to CUPRAC method[1].

Formulations		MIXING		REFINING		CONCHING		TEMPERING		FINAL PRODUCT	
		1	2	1	2	1	2	1	2	1	2
A	1	6884.45	6733.62	8694.41	8609.57	6827.89	6460.24	5621.25	5253.60	6634.48	6434.17
	2	6950.44	6667.63	9014.92	8515.30	7025.85	6365.97	5555.26	5159.33	6327.73	6476.23
	3	6903.30	6648.78	8854.67	8477.59	7431.21	6337.69	5828.64	5149.90	7015.92	6355.81
	Average	6912.73	6683.34	8854.67	8534.15	7094.98	6387.97	5668.38	5187.61	6659.37	6422.07
	Total Average	6798.04 ± 130.54 c		8694.41 ± 207.22 c		6741.48 ± 435.25 c		5428.00 ± 280.71 b		6540.72 ± 256.78 b	
B	1	7959.11	7817.71	9514.55	9429.71	9062.06	8157.08	7110.70	6403.68	7383.77	6914.23
	2	7902.55	7676.31	9618.25	9278.88	9429.71	8006.25	7082.41	6262.28	7350.30	6905.52
	3	7977.97	7780.00	9835.06	9410.85	8892.38	8128.80	6196.29	6384.83	6969.94	6936.54
	Average	7946.55	7758.01	9655.95	9373.15	9128.05	8097.38	6796.47	6350.26	7234.67	6918.76
	Total Average	7852.28 ± 115.87 b		9514.55 ± 193.38 b		8612.71 ± 592.82 b		6573.36 ± 412.59 a		7076.72 ± 226.23 a	
C	1	9985.89	9816.21	11343.36	10240.42	10306.41	10306.41	6639.35	6139.73	6844.33	6575.29
	2	10070.74	9505.12	10890.87	9985.89	9740.79	10042.45	6601.64	6912.73	6612.65	6475.67
	3	9976.47	9665.38	9759.65	10155.58	9514.55	10221.57	7063.56	7091.84	6942.73	6558.23
	Average	10011.03	9662.24	10664.63	10127.30	9853.92	10190.14	6768.19	6714.77	6799.90	6536.40
	Total Average	9836.63 ± 217.38 a		10395,96 ± 599.59 a		10022,03 ± 328.21 a		6741.48 ± 359.97 a		6668,15 ± 182.90 b	

[1]Values represent the means ± standard deviations of 3 replicates from 3 extracts of 2 independent processing events. All contents are expressed as μmol TEAC/100 g dry weight. Different letters in the columns represent statistically significant differences between formulations (P<0.05).

In refining process, antioxidant capacities of formulation A and formulation B had lower values (16% and 8%, respectively) compared to the control sample.

The results of conching process showed that highest total antioxidant activity was obtained in formulation C (10022.03 μmol TEAC/100 g DM), followed by formulation B (8612.71 μmol TEAC/100 g DM) and formulation A (6741.48 μmol TEAC/100 g DM) (Table 4.12). The differences among the total antioxidant capacities of formulations at conching process step were significant ($P<0.05$). Similarly to the DPPH method, antioxidant activity of the formulation A and formulation B pointed out lower values (33% and 14%, respectively) against control samples.

In tempering process, the results demonstrated that highest total antioxidant activity was obtained in formulation C (6741.48 μmol TEAC/100 g DM), followed by formulation B (6573.36 μmol TEAC/100 g DM) and formulation A (5428.00 μmol TEAC/100 g DM) (Table 4.12). The differences among the total antioxidant activities of formulations at tempering process step were found to be significant ($P<0.05$). In tempering process, there were slightly lower values of about 2% obtained for the antioxidant capacity of formulation B against control sample, but a higher difference occurred of about 19% between formulation A and control sample.

According to the results of final products, formulation C had an antioxidant activity of 6668.15 μmol TEAC/100 g DM, whereas formulation B and A had activity values of 7076.72 μmol TEAC/100 g DM and 6540.72 μmol TEAC/100 g DM, respectively. The differences among the total antioxidant capacities of chocolate final products were found to be significant ($P<0.05$).

Additionally, total antioxidant capacities of alkalized cocoa powder, cocoa mass and deskinned almond paste according to CUPRAC method were compared with chocolate final products. Total antioxidant capacity of alkalized cocoa powder ranged from 5535.36 μmol TEAC/100 g DM to 6339.79 μmol TEAC/100 g DM (Table 4.6). In comparison with chocolate final products total antioxidant capacity of chocolate last products were found to be higher than alkalized cocoa powder. Total antioxidant capacity of cocoa mass ranged from 19813.94 μmol TEAC/100 g DM to 22494.31 μmol TEAC/100 g DM (Table 4.6). These values were found to be higher compared to chocolate final products. Total antioxidant capacity of deskinned

almond paste was very low compared to other cocoa product extracts. The decreasing order of the extracts based on the total antioxidant capacities observed with CUPRAC assay: cocoa mass > final products > alkalized cocoa powder >> deskinned almond paste.

There is no available information about the measurement of the antioxidant capacity of chocolate products in the current literature to compare the results of this study. Therefore the results of the chocolate antioxidant capacities observed in this study are new findings in the available literature.

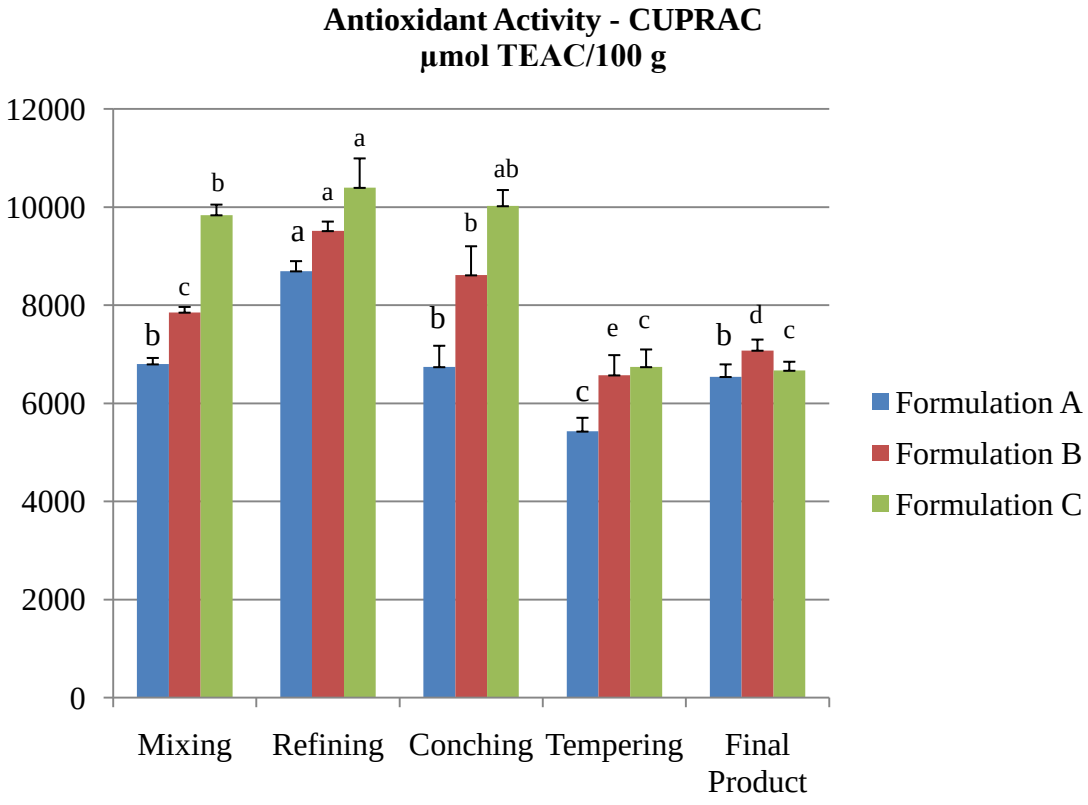

Figure 4.5 : Comparison of total antioxidant capacities of all manufacturing steps of chocolate samples according to CUPRAC method[1].

[1]Different letters on the columns represent statistically significant differences ($p<0.05$).

All total antioxidant capacity values of chocolate formulations according to CUPRAC method were compared to evaluate the effect of processing on chocolate and it was observed that the highest total antioxidant capacity values were obtained from refining steps, followed by mixing, conching and tempering steps. Figure 4.5 shows the average total antioxidant activities of all manufacturing process steps of all

formulations according to CUPRAC method. The antioxidant activity of formulation A increased about 28% in refining process compared to mixing step and decreased in conching and tempering steps about 22% and 19% compared to prior steps, respectively. The antioxidant activity of formulation B pointed out 21% higher values than mixing step and 9% and 24% lower values than their prior steps in conching and refining process, respectively. Formulation C had 6% higher antioxidant capacity values in refining step compared to mixing step. Similar to other formulations, the antioxidant capacity values decreased in following conching and tempering steps by 3% and 32% compared to their prior steps, respectively. The difference of total antioxidant activities of each manufacturing process steps of all chocolate formulations were significant ($P<0.05$).

4.3.3.4. FRAP

Table 4.13 and Figure 4.6 represent the total antioxidant activity of three chocolate formulations in all process steps. The results indicated that highest total antioxidant activity was obtained in formulation C (4834.42 μmol TEAC/100 g DM), followed by formulation B (4361.84 μmol TEAC/100 g DM) and formulation A (3614.12 μmol TEAC/100 g DM) in mixing step (Table 4.13). S

Similarly to the results of ABTS and CUPRAC methods, in FRAP assay, formulation A and formulation B total antioxidant capacities were found to be about 25% and 10% lower, respectively compared to the control sample in mixing process. The differences among the total antioxidant activities of formulations at mixing process step were found to be significant ($P<0.05$).

According to the results of refining process, it was observed that the highest total antioxidant activity was obtained in formulation C (5077.56 μmol TEAC/100 g DM), followed by formulation B (4597.15 μmol TEAC/100 g DM) and formulation A (4458.71 μmol TEAC/100 g DM) (Table 4.13).

Similarly to the CUPRAC method, in refining process the total antioxidant acitivity values of the formulation A and B found to be about 12% and 9% lower than the control sample. The differences among the total antioxidant activities of formulations at refining process step were found to be significant ($P<0.05$).

Table 4.13 : Total antioxidant capacities of chocolate formulations in chocolate manufacturing steps according to FRAP method[1].

Formulations		MIXING		REFINING		CONCHING		TEMPERING		FINAL PRODUCT	
		1	2	1	2	1	2	1	2	1	2
A	1	3624.57	3604.98	4541.43	4388.62	3478.29	3176.59	3023.79	2823.96	3687.60	3656.74
	2	3696.41	3594.53	4508.77	4375.56	3561.88	3162.23	3066.89	2812.20	3486.41	3652.15
	3	3811.34	3352.91	4564.93	4372.94	3503.11	3158.31	3111.29	2809.59	3850.82	3591.81
	Average	3710.77	3517.48	4538.38	4379.04	3514.43	3165.71	3067.32	2815.25	3674.94	3633.57
	Total Average	3614.12 ± 151.31 c		4458.71 ± 89.24 c		3340.07 ±193.02 c		2941.29 ±140.89 c		3654.25 ±119.86 a	
B	1	4404.29	4318.09	4668.11	4540.12	4430.41	3921.05	3607.59	3368.59	4199.62	3816.32
	2	4410.82	4297.19	4661.58	4517.92	4391.23	3901.46	3603.68	3348.99	4140.97	3777.23
	3	4429.10	4311.56	4660.28	4534.90	4382.09	3915.82	3569.72	3365.97	3914.36	3932.92
	Average	4414.74	4308.95	4663.32	4530.98	4401.24	3912.78	3593.66	3361.18	4084.98	3841.49
	Total Average	4361.84 ± 58.90 b		4597.15 ± 72.91 b		4157.01 ± 268.11 b		3477.42 ± 128.19 b		3963.24 ± 171.78 b	
C	1	4861.41	4625.01	5200.99	4961.98	4805.25	5028.59	4002.02	4137.85	3906.78	3580.77
	2	4890.14	4747.78	5214.05	4924.10	4820.92	4993.32	3965.45	4102.59	3691.89	3542.96
	3	5112.17	4769.99	5212.74	4951.53	4913.65	5015.52	3891.01	4126.10	3842.99	3715.45
	Average	4954.58	4714.26	5209.26	4945.87	4846.60	5012.48	3952.83	4122.18	3813.89	3613.06
	Total Average	4834.42 ± 165.22 a		5077.56 ± 144.86 a		4929.54 ±98.77 a		4037.50 ± 100.07 a		3459.57 ±142.34 a	

[1]Values represent the means ± standard deviations of 3 replicates from 3 extracts of 2 independent processing events. All contents are expressed as µmol TEAC/100 g dry weight. Different letters in the columns represent statistically significant differences between formulations (P<0,05).

The results obtained from conching step also demonstrated that the highest total antioxidant activity was obtained in formulation C (4929.54 μmol TEAC/100 g DM), followed by formulation B (4157.01 μmol TEAC/100 g DM) and formulation A (3340.07 μmol TEAC/100 g DM) (Table 4.13). Similarly to the DPPH and CUPRAC methods, antioxidant capacities of formulation A and B were about 32% and 16% lower than the control sample, respectively. The differences among the total antioxidant capacities of formulations at conching process step were found to be significant ($P<0.05$).

In tempering step, it was observed that highest total antioxidant activity was obtained in formulation C (4037.50 μmol TEAC/100 g DM), followed by formulation B (3477.42 μmol TEAC/100 g DM) and formulation A (2941.29 μmol TEAC/100 g DM) (Table 4.13). Similarly to the ABTS and DPPH methods, in tempering process the antioxidant capacities of formulation A and formulation B were 27% and 14% lower than the control sample, respectively. The differences among the total antioxidant activities of formulations at tempering process step were found to be significant ($P<0.05$).

According to the results of final products, formulation C had an antioxidant activity of 3459.57 μmol TEAC/100 g DM, whereas formulation B and A had activity values of 3963,24 μmol TEAC/100 g DM and 3654.25 μmol TEAC/100 g DM, respectively (Table 4.13). The differences among the total antioxidant activities of chocolate final products were found to be significant ($P<0.05$).

Additionally, total antioxidant capacities of alkalized cocoa powder, cocoa mass and deskinned almond paste according to FRAP method were compared with chocolate final products. Total antioxidant capacity of alkalized cocoa powder ranged from 1733.84 μmol TEAC/100 g DM to 1986.78 μmol TEAC/100 g DM (Table 4.7). In comparison with chocolate final products total antioxidant capacity of chocolate last products were found to be higher than alkalized cocoa powder. Total antioxidant capacity of cocoa mass ranged from 11157.80 μmol TEAC/100 g DM to 12655.41 μmol TEAC/100 g DM (Table 4.7). These values were found to be higher compared to chocolate final products. Total antioxidant capacity of deskinned almond paste was very low compared to other cocoa product extracts. The decreasing order of the

extracts based on their total antioxidant capacities observed with FRAP assay: cocoa mass > final products > alkalized cocoa powder >> deskinned almond paste.

It was suggested according to our results that almond addition to chocolate formulations decrease total antioxidant capacity as a result of flavonoid-protein binding. Serafini et al. (2009) studied the effect of *in vitro* addition of milk to blueberry extracts on the antioxidant activity. The *in vitro* addition of milk to blueberry extracts resulted in a precipitation of blueberry antioxidants. This finding is in agreement with the data of Langley-Evans (2000) that showed reduction *in vitro* antioxidant capacity of black tea according to FRAP method with the addition of whole milk (28% reduction), semiskimmed milk (22% reduction) and skimmed milk (12% reduction).

In another study (Ryan and Petit, 2010) about protein effects on phenolics it was investigated that the addition of 10, 15, and 20 mL of semiskimmed or skimmed milk significantly ($P < 0.05$) decreased the total antioxidant capacity compared to tea with the same volume of water added according to the FRAP method. The addition of whole milk to each of the teas also decreased the total antioxidant capacity but to a lesser extent when compared to semiskimmed and skimmed milk. Besides, antioxidant activity was decreased with increased amount of milk added. This study also proved polyphenol binding to proteins and that the interaction between the flavonoids and proteins affects their antioxidant capacity.

Komes et al. (2009) studied the antioxidant capacities of dark chocolate samples with 88% cocoa solids, 72% cocoa solids and 60% cocoa solids. Comparing our data with the results obtained by Komes et al. (2009) which were 2493 μmol TEAC / 100 g, 1640 μmol TEAC / 100 g and 1593 μmol TEAC / 100 g, the results presented in our study was higher. These variations between two studies might be a result of different cocoa beans used in chocolate manufacturing, different process conditions and different storage time and conditions.

All total antioxidant capacity values of chocolate formulations according to FRAP method were compared to evaluate the effect of processing on chocolate and it was observed that the highest total antioxidant capacity values were obtained from refining steps, followed by mixing, conching and tempering steps. Figure 4.6

represents the average total antioxidant activities of all manufacturing process steps of all formulations according to FRAP method.

In refining step, the increase compared to mixing step for antioxidant capacities of formulations A, B and C observed about 23%, 5% and 5%, respectively. In conching step, lower antioxidant capacity values were observed (about 25%, 9% and 3% for formulation A, B and C, respectively) compared to refining step. In tempering process, about 12%, 16% and 18% lower antioxidant capacity values were detected compared to conching step, for formulations A, B and C, respectively.

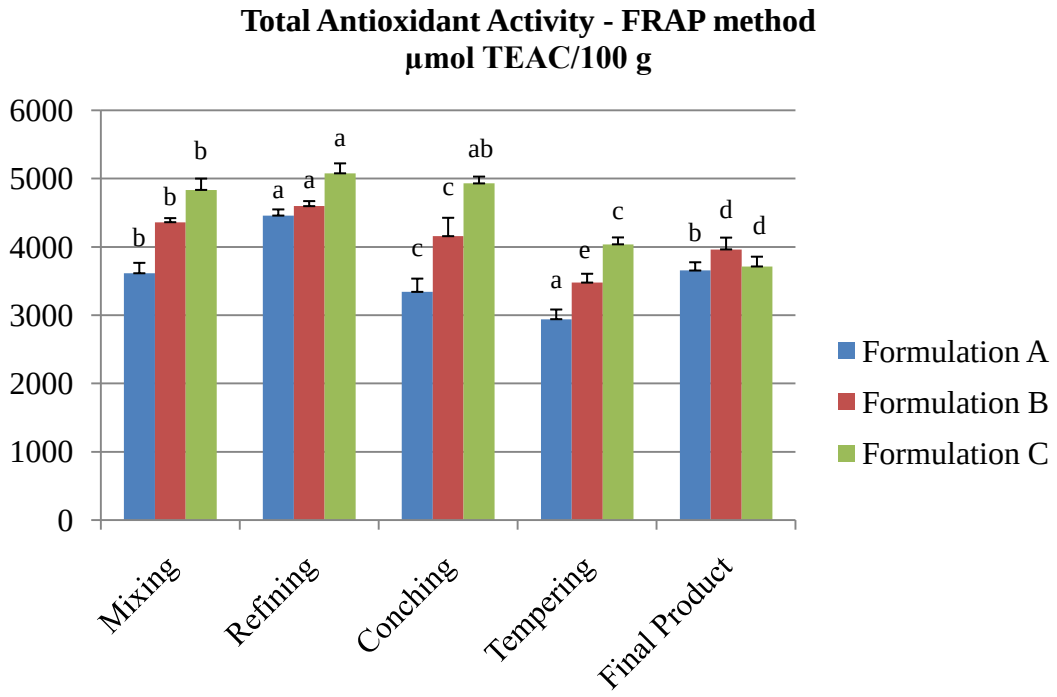

Figure 4.6 : Comparison of total antioxidant capacities of all manufacturing steps of chocolate samples according to FRAP method[1].

Different letters on the columns represent statistically significant differences ($p<0.05$).

4.3.3.5. Comparison of antioxidant capacity methods

All the antioxidant capacity measurement methods of ABTS, DPPH, CUPRAC and FRAP gave a similar trend at the end of the comparison between three formulations, although the observed values were different according to the methods. In all the methods control sample gave the highest antioxidant capacity, followed by formulation B and formulation A. The results of the antioxidant capacities were also represented similar trends with the total flavonoid contents and the total phenolic

contents of all the samples. Generally in all formulations, the differences between all antioxidant capacity methods were found to be significant (P<0.05).

The results of all the methods for antioxidant capacity measurements were compared for formulation A, B and C in Figure 4.7, Figure 4.8 and Figure 4.9.

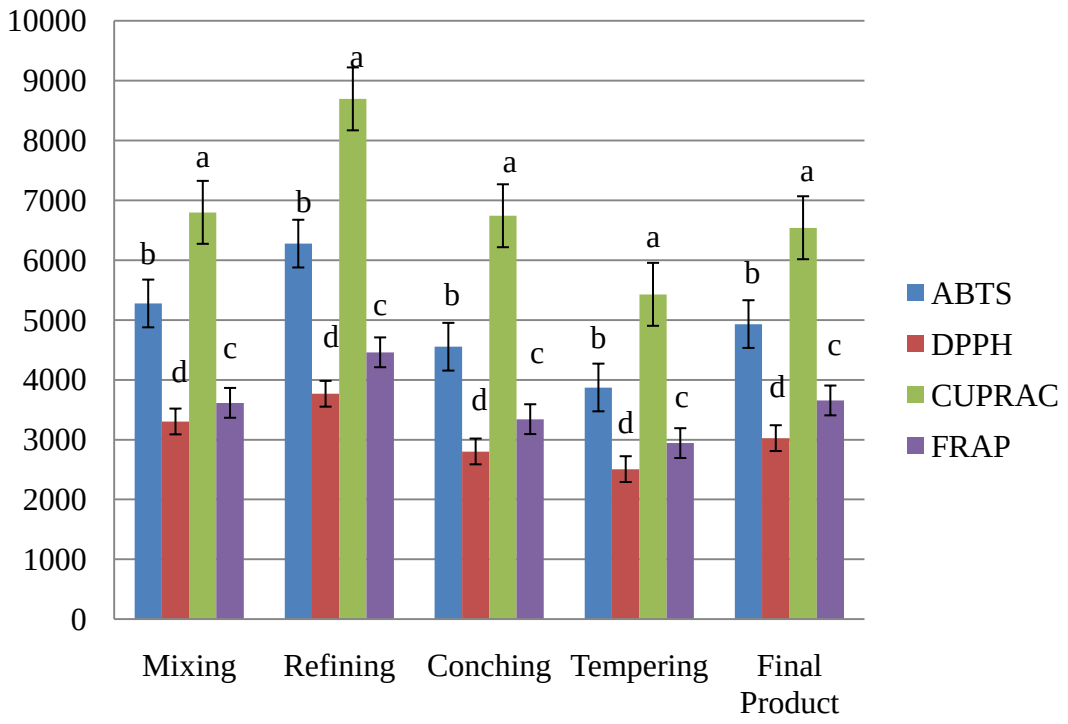

Figure 4.7 : Comparison of antioxidant capacity methods for formulation A[1].

[1]Different letters on the columns represent statistically significant differences (p<0.05).

The highest antioxidant capacity values were observed using CUPRAC method and the lowest antioxidant capacity values were observed using DPPH method for formulation A. ABTS method also gave higher values, but FRAP method resulted with lower activity values in comparison with ABTS and CUPRAC methods. The differences between the results of all the antioxidant capacity methods were found to be significant (P<0.05).

The highest antioxidant capacity values were observed in CUPRAC method, followed by ABTS, FRAP and DPPH methods for formulation B, respectively. The differences between the results of all the antioxidant capacity methods were found to be significant for formulation B (P<0.05).

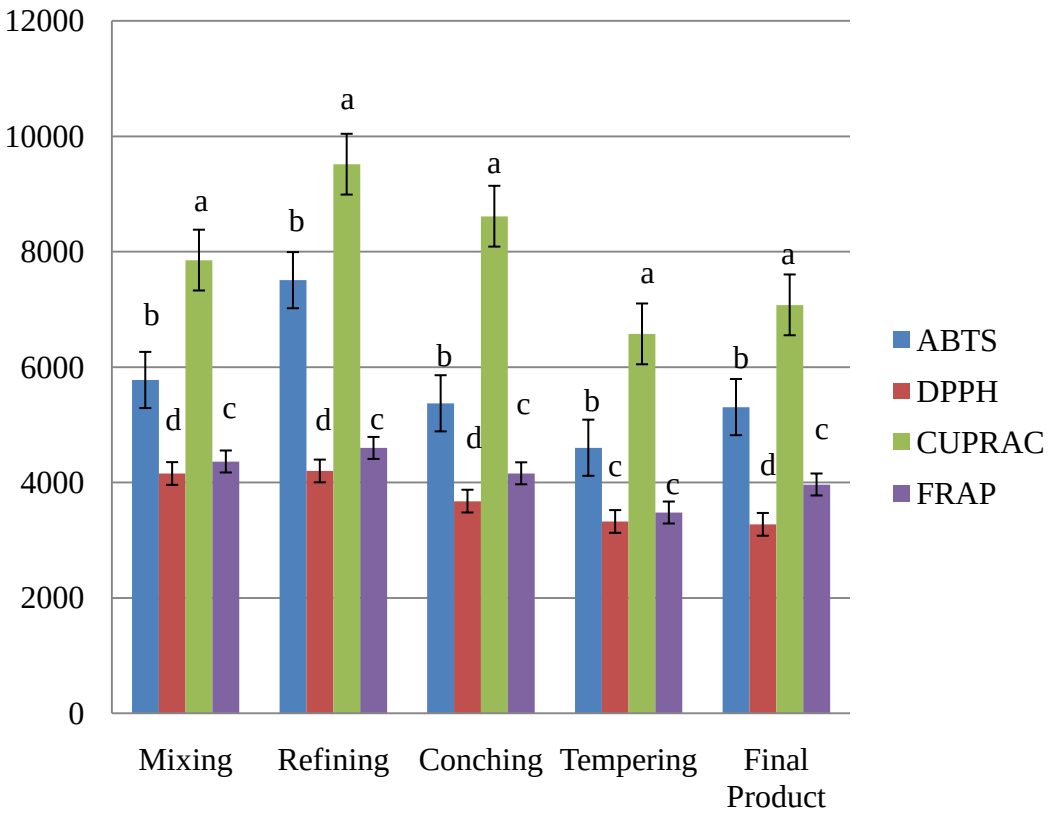

Figure 4.8 : Comparison of antioxidant capacity methods for formulation B[1].

[1]Different letters on the columns represent statistically significant differences ($p<0.05$).

Similarly to formulations A and B, highest antioxidant capacity values were observed using CUPRAC method and the lowest values were observed using DPPH method for formulation C. The differences between the results of all the antioxidant capacity methods were found to be significant for formulation B ($P<0.05$).

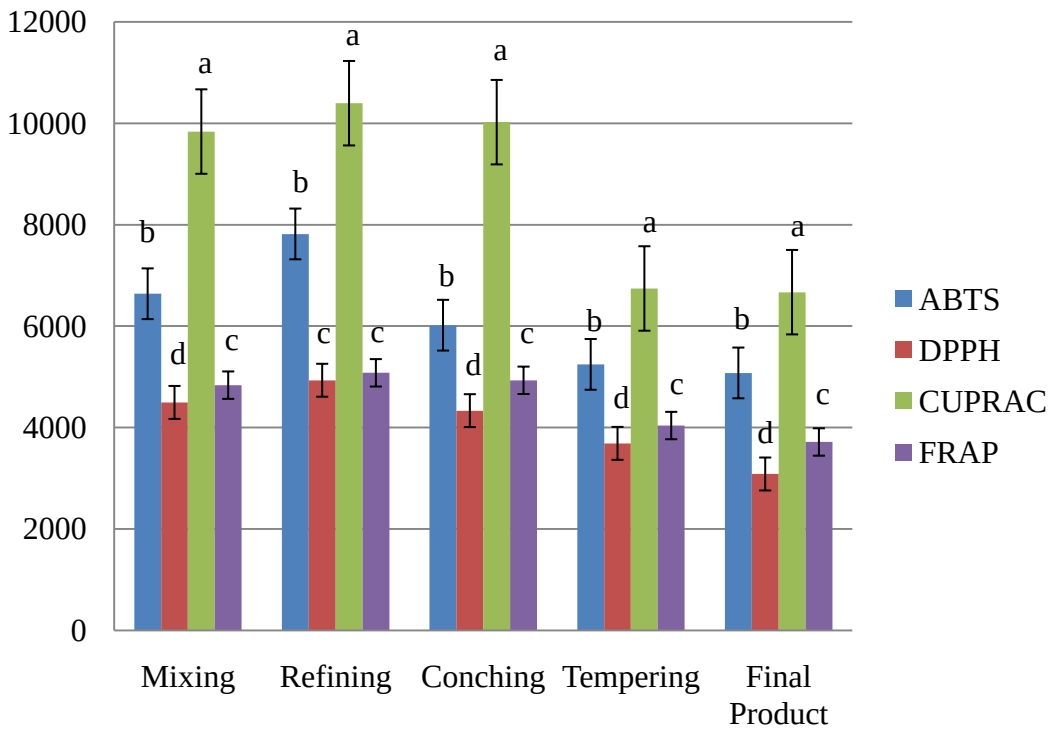

Figure 4.9 : Comparison of antioxidant capacity methods for formulation C[1].

[1]Different letters on the columns represent statistically significant differences ($p<0.05$).

Comparing the results of the applied antioxidant capacity assays on chocolate formulations, it can be noticed that CUPRAC and ABTS methods exhibit high antioxidant capacity values whereas the efficiency of FRAP and DPPH methods were lower with regard to CUPRAC and ABTS methods. Although ABTS and DPPH methods work with the same principle, the results vary significantly. The antioxidant capacities of chocolate extracts obtained by ABTS assay was higher than the results obtained by DPPH assay. The difference between the results of two antioxidant capacity assays becomes more obvious considering the fact that DPPH radical reacts only with lipophilic antioxidants while ABTS reacts with both lipophilic and hydrophilic antioxidants (Prior et al., 2005). Kim et al. (2002) and Arnao (2000) reported that these differences may be due to absorbance interruption at 517 nm by other compounds in the DPPH assay. The results of this study are in full agreement with the study of Komes et al. (2009) studied antioxidant capacity of various commercial cocoa products using ABTS, DPPH and FRAP methods. They also reported that DPPH method resulted lower antioxidant capacity values than ABTS method. Dykes et al. (2005) also studied the antioxidant capacity of sorghum

using ABTS and DPPH methods. They found out that DPPH values were lower than the ABTS values for all sorghum samples. They suggested that it was because of the fact that anthocyanins cause interference leading to underestimation of antioxidant capacity when using DPPH assay (Dykes et al., 2005).

4.3.4. Correlations between total phenolics, flavonoids and antioxidant capacities

The correlations between the total phenolic contents, total flavonoid contents and total antioxidant capacities according to ABTS, DPPH, CUPRAC and FRAP methods of chocolate samples were investigated. The Pearson's correlation coefficients are represented in Table 4.14.

In mixing process, the highest correlation coefficient was obtained between total flavonoid and antioxidant activity according to FRAP method (r = 0.988) and the lowest correlation coefficient value was found to be between total phenolic and antioxidant activity according to ABTS method (r = 0.801) (Table 4.14).

In refining process, the highest correlation coefficient (r = 0.987) was obtained between total phenolics and antioxidant capacity according to DPPH method and the lowest correlation was found to be between total flavonoids and antioxidant capacity according to ABTS method (r = 0.740) (Table 4.14).

In conching process, the highest correlation coefficient was obtained between antioxidant capacity methods FRAP and DPPH (r = 0.995) and the lowest correlation was obtained from total phenolics and antioxidant capacity according to ABTS method (r = 0.915) (Table 4.14).

The highest correlation coefficient was obtained between antioxidant capacity methods FRAP and ABTS results (r = 0.979) whereas the lowest value was observed between antioxidant capacity methods CUPRAC and FRAP (r = 0.832) methods in tempering process (Table 4.14).

The lowest correlation coefficient was observed between antioxidant capaciry according to FRAP and ABTS methods (r = 0.754) while lowest value was observed between antioxidant capacity according to DPPH and CUPRAC (r = 0.983) in final products (Table 4.14).

Table 4.14 : Correlations among chocolate antioxidant capacities, total phenolics and flavonoids

		ABTS	DPPH	CUPRAC	FRAP	TOTAL PHENOLIC	TOTAL FLAVONOID
MIXING	ABTS		0.863*	0.956*	0.903*	0.801*	0.914*
	DPPH	0.863*		0.906*	0.971*	0.969*	0.977*
	CUPRAC	0.956*	0.906*		0.939*	0.818*	0.953*
	FRAP	0.903*	0.971*	0.939*		0.938*	0.988*
	TOTAL PHENOLIC	0.801*	0.969*	0.818*	0.938*		0.938*
	TOTAL FLAVONOID	0.914*	0.977*	0.953*	0.988*	0.938*	
REFINING	ABTS		0.860*	0.91*	0.775*	0.891*	0.740*
	DPPH	0.860*		0.912*	0.973*	0.987*	0.974*
	CUPRAC	0.910*	0.912*		0.889*	0.914*	0.871*
	FRAP	0.775*	0.973*	0.889*		0.953*	0.978*
	TOTAL PHENOLIC	0.891*	0.987*	0.914*	0.953*		0.943*
	TOTAL FLAVONOID	0.740*	0.974*	0.871*	0.978*	0.943*	
CONCHING	ABTS		0.930*	0.942*	0.927*	0.915*	0.934*
	DPPH	0.930*		0.990*	0.995*	0.974*	0.972*
	CUPRAC	0.942*	0.990*		0.985*	0.968*	0.991*
	FRAP	0.927*	0.995*	0.985*		0.989*	0.971*
	TOTAL PHENOLIC	0.915*	0.974*	0.968*	0.989*		0.962*
	TOTAL FLAVONOID	0.934*	0.972*	0.991*	0.971*	0.962*	
TEMPERING	ABTS		0.939*	0.846*	0.979*	0.974*	0.906*
	DPPH	0.939*		0.894*	0.952*	0.978*	0.932*
	CUPRAC	0.846*	0.894*		0.832*	0.908*	0.868*
	FRAP	0.979*	0.952*	0.832*		0.974*	0.917*
	TOTAL PHENOLIC	0.974*	0.978*	0.908*	0.974*		0.951*
	TOTAL FLAVONOID	0.906*	0.932*	0.868*	0.917*	0.951*	
FINAL PRODUCT	ABTS		0.857*	0.903*	0.754*	0.837*	0.939*
	DPPH	0.857*		0.983*	0.974*	0.976*	0.976*
	CUPRAC	0.903*	0.983*		0.954*	0.972*	0.981*
	FRAP	0.754*	0.974*	0.954*		0.952*	0.904*
	TOTAL PHENOLIC	0.837*	0.976*	0.972*	0.952*		0.950*
	TOTAL FLAVONOID	0.939*	0.976*	0.981*	0.904*	0.950*	

*$r \geq 0.7$

Comparing the total phenolic contents, total flavonoid contents and antioxidant capacities of chocolate samples in all manufacturing process steps, it was suggested that these values were highly correlated. These results were also in agreement with Othman et al. (2007) who found out strong correlations between FRAP assay and phenolic content for both the ethanolic (r = 0.764) and aqueous extracts (r = 0.782) of different varieties of cocoa beans. Benzie and Stezo (1999) also found a strong correlation between total phenolic content and FRAP assay. Gardner et al. (2000) analyzed the synthetic free radical potassium nitrodisulfonate and Fe^{3+} using FRAP method and found strong correlation with phenolic content.

It was reported that phenolic compounds have redox properties which allow them to act as reducing agents, singlet oxygen quenchers and hydrogen donators. Therefore, phenolic compounds play an important role in determining the antioxidant capacity (Rice-Evans et al., 1997). In this study, the correlation between the total phenolic content and antioxidant capacities were found to be high. These findings suggest that total phenolic content is a good predictor of *in vitro* antioxidant capacity.

The study of Dykes et al. (2005) is in agreement with our findings that they found out high correlations between ABTS and DPPH methods, although DPPH results were significantly lower than ABTS results. They also reported high correlations between total phenolics and antioxidant capacity (total phenolics vs. ABTS, r = 0.97 and total phenolics vs. DPPH, r = 0.97).

4.4. Sensory Analysis of Chocolate Final Products

The panelists agreed upon a total of 20 different descriptive terms in chocolate samples and the data of the evaluation of panelists were analyzed. In this study, Kruskal-Wallis test was used to analyze the sensory analysis data of two sensory panels to determine if there were any differences for pre-determined descriptives between different chocolate samples tested by panelists. The Kruskal-Wallis test is a nonparametric test used for measuring the equality of medians of groups. Table 4.15 displays the results of the Kruskal-Wallis test for all descriptives of chocolate samples.

Table 4.15 : Kruskal-Wallis test statistics for all descriptives of chocolate samples[1,2].

DESCRIPTIVES	Cocoa Odor	Almond Odor	Vanillin Odor	Cocoa Aroma	Vanillin Aroma
Chi-Square	4.173	24.331	1.900	5.311	0.255
df	2	2	2	2	2
Asymp. Sig.	0.124	**0.000***	0,387	0.070	0.880
DESCRIPTIVES	Almond Aroma	Alkaline Taste	Sweetness	Bitter	Oily taste
Chi-Square	14.851	1.053	4.708	6.077	18.213
df	2	2	2	2	2
Asymp. Sig.	**0.001***	0.591	0.095	**0.048***	**0.000***
DESCRIPTIVES	Earthiness	Melting rate	Firmness	Brittleness	Slippery
Chi-Square	0.136	7.267	8.514	2.013	5.632
df	2	2	2	2	2
Asymp. Sig.	0.934	**0.026***	**0.014***	0.366	0.060
DESCRIPTIVES	Adhesiveness	Oily mouthfeel	Water absorbtion	Disappearance	Puckery
Chi-Square	4.309	13.455	4.139	4.891	3.280
df	2	2	2	2	2
Asymp. Sig.	0.116	**0.001***	0.126	0.087	0.194

[1]Data represents the results of 2 independent sensory analysis panels (N=12)
[2]Grouping variable: Treatment
*p<0.05

There were statistically significant differences between three formulations of chocolate for the almond odor (H(2) = 24.331, p = 0.000), almond aroma (H(2) = 14.851, p = 0.001), bitter (H(2) = 6.077, p = 0.048), oily taste H(2) = 18.213, p = 0.000), melting rate (H(2) = 7.267, p = 0.026), firmness (H(2) = 8.514, p = 0.014, oily mouthfeel (H(2) = 13.455, p = 0.001) (Table 4.15).

The almond odor is the only odor characteristic that was found to be significantly different between chocolate samples by the panelists (P<0.05). It was suggested that addition of almond to chocolate formulations can be detected by smelling the chocolate products without breaking. Besides, almond aroma is the only aroma characterictic that was found to be significantly different between chocolate samples by the panelists (P<0.05). The addition of almond to chocolate formulation can also be detected by panelists while chewing the products by pushing the air in the oral cavity to the nasal cavity. The differences of bitter taste and oily taste were also found to be significant between chocolate samples (Table 4.15). The differences of texture characteristics such as melting rate, oily mouthfeel and firmness were found to be significant (P<0.05) according to different almond contents in chocolate (Table 4.15). The level of melting of different chocolate in the hand were significantly

different ($P<0.05$). Firmness, which is described as amount of force needed to bite through a piece of chocolate by incissors were significantly different ($P<0.05$) between three chocolate products. The amount of oil that surrounds the mouth while chewing was also significantly different ($P<0.05$) between products. It was also suggested that the significant differences of oily taste, melting rate, firmness and oily mouhfeel descriptives can be affected by addition of almond content since almond contains high percentage of total lipids. According to USDA Nutrient Database, almonds have 49.42% of total lipid content (USDA, 2010).

There was no significant difference detected for the vanillin and cocoa odor between chocolate samples. Three chocolate formulations contained same contents of cocoa solids and vanillin, but different contents of almond, so that it was correctly detected by smelling. Cocoa aroma and vanillin aroma were found to be insignificant (Table 4.15). Alkaline taste and earth taste were also found to be insignificant between the chocolate samples (Table 4.15). Although three chocolate samples contained different amounts of sugar, there was no difference in sweetness property. It was suggested that the difference of sweetness might be concealed by the intensity of bitter taste. The differences of texture descriptives such as brittleness, slippery, adhesiveness, water absorption and disappearance in mouth were also found to be insignificant between chocolate samples (Table 4.15).

5. CONCLUSION

Health promoting effects of cocoa and cocoa products like dark chocolate arise from cocoa polyphenols and their antioxidant capacity with an increased interest of scientists all around the world on this subject. However, less attention has been dedicated to the effect of processing on chocolate phenolic and flavonoid contents and antioxidant capacity. Besides, there is no available information on the effect of almond content on phenolic and flavonoid contents and antioxidant capacity of dark chocolate.

In this study, the effect of almond content on dark chocolate phenolic and flavonoid contents and antioxidant capacity were analyzed for three different formulations of chocolate in all manufacturing steps. As a conclusion of these studies, it was observed that the highest total phenolic and flavonoid contents as well as antioxidant capacity were observed in control sample with no almonds in all the manufacturing steps. It was followed by formulation B with 7.5% almond content and formulation A with 15% almond content. It was suggested that the almond content might decrease the total phenolic and flavonoid contents and antioxidant capacity due to polyphenol interactions with proteins.

It is expected that a single method cannot determine all the antioxidant compounds available in the food matrix correctly. All the methods have their own advantages and disadvantages. Even the results of the methods sharing the same principle like ABTS and DPPH can show important differences in their response to antioxidants. It is highly recommended to apply several test procedures to evaluate antioxidant capacity. Therefore, in this study four different types of antioxidant capacity analysis methods ABTS, DPPH, CUPRAC and FRAP were performed. As a conclusion to all the antioxidant activity values, it was observed that CUPRAC method yielded consistent data and highest antioxidant activity values with respect to the results of ABTS, DPPH and FRAP methods. Only the CUPRAC and ABTS methods yielded sufficiently high TEAC values in the analyzed samples of chocolate processing steps. The antioxidant capacity values observed by DPPH method were found to be lowest amongst all other methods. It was suggested that the result of this low antioxidant

capacity values using DPPH method compared to other antioxidant capacity measurement methods can be explained by the fact that DPPH reacts only with lipophilic antioxidants whereas ABTS reacts both with hydrophilic and lipophilic antioxidants. It can also be explained by the absorbance interaction at 517 nm by other compounds or interference of anthocyanins leading to underestimation of antioxidant capacity.

The results of correlation analysis were high indicating that there is a strong correlation between the total phenolic content, total flavonoid content and antioxidant capacity values according to ABTS, DPPH, CUPRAC and FRAP methods for every manufacturing steps of all the formulations. It was suggested that the antioxidant capacity of chocolate products could be contributed to the phenolic compounds especially flavonoids.

The results of the choice of extraction solvent indicated that in comparison with 70% methanol, 80% methanol is a more efficient solvent to extract phenolic and flavonoid compounds and antioxidants from cocoa beans

Another aim of this study was to evaluate the effect of chocolate manufacturing process steps on phenolic and flavonoid contents and antioxidant capacity of all chocolate formulations. It was observed that refining process yielded higher values compared to the mixing process. It was suggested that this increase might be resulted from the decreased particle size. Reduction of particle size might break up certain structures of food matrix and released bound antioxidants. Moreover, particle surface enlargement might increase solvent penetration. Although particle size reduction in refining process increased *in vitro* antioxidant capacity, it also reduced thermal stability. The decrease of the total phenolic and flavonoid contents and total antioxidant capacity in the following conching and tempering steps might be resulted from degradation of some antioxidant compounds according to the reduced thermal stability. There were statistically significant differences between all the manufacturing steps in all formulations. Generally, the highest values were obtained in refining step, followed by mixing, conching and tempering steps.

As a conclusion of this study, it was observed that both the almond content and process conditions affect the *in vitro* total phenolic and flavonoid contents and antioxidant capacity significantly.

The process conditions should be optimized to minimize the decrease of the total phenolic and flavonoid contents and antioxidant capacity for a healthier chocolate.

By controlling the process during chocolate manufacturing, chocolate having a high-flavonoid content can be produced.

The interactions of chocolate components with almonds, which decreased the total phenolic content, total flavonoid content and antioxidant capacity needed to be investigated to understand the exact mechanisms of chocolate polyphenol-protein interactions. In addition, future studies should be focused on the bioavailability of chocolate polyphenols bound to proteins.

In addition to *in vitro* studies, future clinical studies investigating the bioavailability of phenolic compounds would provide valuable data for elucidating the effect of food processing on human health. Besides, in vivo studies should be conducted to evaluate the phenolic compounds functionality to obtain if the same amount of antioxidants would be able to offer similar health benefits.

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APPENDICES

APPENDIX A : Production Recipes

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APPENDIX A : Production Recipes

Table A.1 : Production recipe for formulation A

Dark chocolate with 65% cocoa and 15% almond content					
CONCHING TIME:	**8 HOURS**				
PRODUCTION TYPE: PILOT SCALE					
INGREDIENTS	**%**	**LIPID %**	**BATCH**		
Sugar	19.00		950.00	COCOA DRY MATTER	
Cocoa butter	20.40	20.40	1020.00	65.40	
Natural cocoa liquor	30.00	16.50	1500.00		
Almond paste	15.00	7.50	750.00		
Nonfat milk powder	0.00		0.00		
Alkalized cocoa powder	15.00	1.65	750.00		
Oil	0.00	0.00	0.00		
Lecithin	0.30	0.30	15.00		
Vanillin	0.10		5.00		
PST	0.00		0.00		
PGPR (E 476)	0.20	0.20	10.00		
TOTAL	100.00	46.55	5000.00		
MIXER					
	GR	**%**	**LIPID%**		**BATCH**
Sugar	950.00	24.05			1045.00
Cocoa butter	0.00	0.00	0.00		0.00
Natural Cocoa Liquor	1500.00	37.97	20.89		1650.00
Almond Paste	750.00	18.99	9.49		825.00
Nonfat milk powder	0.00	0.00			0.00
Alkalized milk powder	750.00	18.99	2.09		825.00
Oil	0.00	0.00	0.00		0.00
Whey powder	0.00	0.00			0.00
TOTAL	3950.00	100.00	32.47		4345.00
CONCHE					
FIRST STEP	**GR**	**%**	**LIPID%**		
FLEX	3950.00	79.00	25.65	31.27	
	150.00	3.00	3.00	3.66	
	1.00	0.02	0.02	0.02	
				34.95	first lipid rate at conche
LAST STEP					
Cocoa Butter	870.00	17.40	17.40		
Lecithin	14.00	0.28	0.28		
Vanillin	5.00	0.10	0.00		
PGPR (E 476)	10.00	0.20	0.20		
TOTAL	5000.00	100.00	46.55		
Thickness:	18 µm				

Table A.2 : Production recipe for formulation B

Dark chocolate with 65% cocoa and 7.5% almond content				
CONCHING TIME: 8 HOURS				
PRODUCTION TYPE: PILOT SCALE				
INGREDIENTS	**%**	**LIPID %**	**BATCH**	
Sugar	26.50		1325.00	COCOA DRY MATTER
Cocoa butter	20.40	20.40	1020.00	65.40
Natural cocoa liquor	30.00	16.50	1500.00	
Almond paste	7.50	3.75	375.00	
Nonfat milk powder	0.00		0.00	
Alkalized cocoa powder	15.00	1.65	750.00	
Oil	0.00	0.00	0.00	
Lecithin	0.30	0.30	15.00	
Vanillin	0.10		5.00	
PST	0.00		0.00	
PGPR (E 476)	0.20	0.20	10.00	
TOTAL	100.00	42.80	5000.00	
MIXER				
	GR	**%**	**LIPID%**	**BATCH**
Sugar	1325.00	33.54		1458.00
Cocoa butter	0.00	0.00	0.00	0.00
Natural Cocoa Liquor	1500.00	37.97	20.89	1650.00
Almond Paste	375.00	9.49	4.75	413.00
Nonfat milk powder	0.00	0.00		0.00
Alkalized milk powder	750.00	18.99	2.09	825.00
Oil	0.00	0.00	0.00	0.00
Whey powder	0.00	0.00		0.00
TOTAL	3950.00	100.00	27.72	4346.00
CONCHE				
FIRST STEP	**GR**	**%**	**LIPID%**	
FLEX	3950.00	79.00	21.90	26.70
	150.00	3.00	3.00	3.66
	1.00	0.02	0.02	0.02
First lipid rate at conche				**30.38**
LAST STEP				
Cocoa Butter	870.00	17.40	17.40	
Lecithin	14.00	0.28	0.28	
Vanillin	5.00	0.10	0.00	
PGPR (E 476)	10.00	0.20	0.20	
TOTAL	5000.00	100.00	42.80	
Thickness:	18 µm			

Table A.3 : Production recipe for formulation C

Dark chocolate with 65% cocoa and 0% almond content					
CONCHING TIME:	**8 HOURS**				
	PRODUCTION TYPE: PILOT SCALE				
INGREDIENTS	**%**	**LIPID %**	**BATCH**		
Sugar	34.00		1700.00	COCOA DRY MATTER	
Cocoa butter	20.40	20.40	1020.00	65.40	
Natural cocoa liquor	30.00	16.50	1500.00		
Almond paste	0.00	0.00	0.00		
Nonfat milk powder	0.00		0.00		
Alkalized cocoa powder	15.00	1.65	750.00		
Oil	0.00	0.00	0.00		
Lecithin	0.30	0.30	15.00		
Vanillin	0.10		5.00		
PST	0.00		0.00		
PGPR (E 476)	0.20	0.20	10.00		
TOTAL	100.00	39.05	5000.00		
MIXER					
	GR	**%**	**LIPID%**		**BATCH**
Sugar	1700.00	41.77			1870.00
Cocoa butter	120.00	2.95	0.00		132.00
Natural Cocoa Liquor	1500.00	36.86	20.89		1650.00
Almond Paste	0.00	0.00	9.49		0.00
Nonfat milk powder	0.00	0.00			0.00
Alkalized milk powder	750.00	18.43	2.09		825.00
Oil	0.00	0.00	0.00		0.00
Whey powder	0.00	0.00			0.00
TOTAL	4070.00	100.00	32.47		4477.00
CONCHE					
FIRST STEP	**GR**	**%**	**LIPID%**		
FLEX	4070.00	81.40	20.55	24.06	
	200.00	4.00	4.00	4.68	
	1.00	0.02	0.02	0.02	
				28.76	first lipid rate at conche
LAST STEP					
Cocoa Butter	700.00	14.00	14.00		
Lecithin	14.00	0.28	0.28		
Vanillin	5.00	0.10	0.00		
PGPR (E 476)	10.00	0.20	0.20		
TOTAL	5000.00	100.00	39.05		
Thickness:	18 μm				

APPENDIX B : Conching process at a laboratory scale conche

Table B.1 : Laboratory conche steps

Step	No	Description	P1	E1	P2	E2
1	0	Step time	10	minutes		
	54	Enter the desired water temperature	45	°C		
	36	Addition	1	Cocoa butter	150,0	GR
	36	Addition	3	Lecithin	1,0	GR
	7	Right rotation speed	1200	RPM		
2	0	Step time	15	minutes		
	55	Enter the desired chocolate temperature	65	°C		
	8	Left rotation speed	1300	RPM		
3	0	Step time	100	minutes		
	55	Enter the desired chocolate temperature	70	°C		
	8	Left rotation speed	1450	RPM		
6	0	Step time	100	minutes		
	55	Enter the desired chocolate temperature	70	°C		
	8	Left rotation speed	1750	RPM		
7	0	Step time	60	minutes		
	55	Enter the desired chocolate temperature	70	°C		
	8	Left rotation speed	1900	RPM		
8	0	Step time	20	minutes		
	55	Enter the desired chocolate temperature	70	°C		
	8	Left rotation speed	2100	RPM		
9	0	Step time	15	minutes		
	55	Enter the desired chocolate temperature	45	°C		
	7	Right rotation speed	2000	RPM		
10	0	Step time	10	minutes		
	55	Enter the desired chocolate temperature	45	°C		
	7	Right rotation speed	2000	RPM		
	36	Addition	10	minutes		
	36	Addition	45	°C		
	36	Addition	2000	RPM		
	36	Addition	3	Lecithin	14.0	GR
11	0	Step time	1	Cocoa butter	870.0	GR
	54	Enter the desired water temperature	4	Polyglycerol Polyricinoleate (PGPR),	10.0	GR
	7	Right rotation speed	2	Vanillin	5.0	GR
	16	Stop	5	minutes		
			45	°C		
			750	RPM		
		Time Duration	335	minutes	5.58	hours

APPENDIX C : Calibration curves

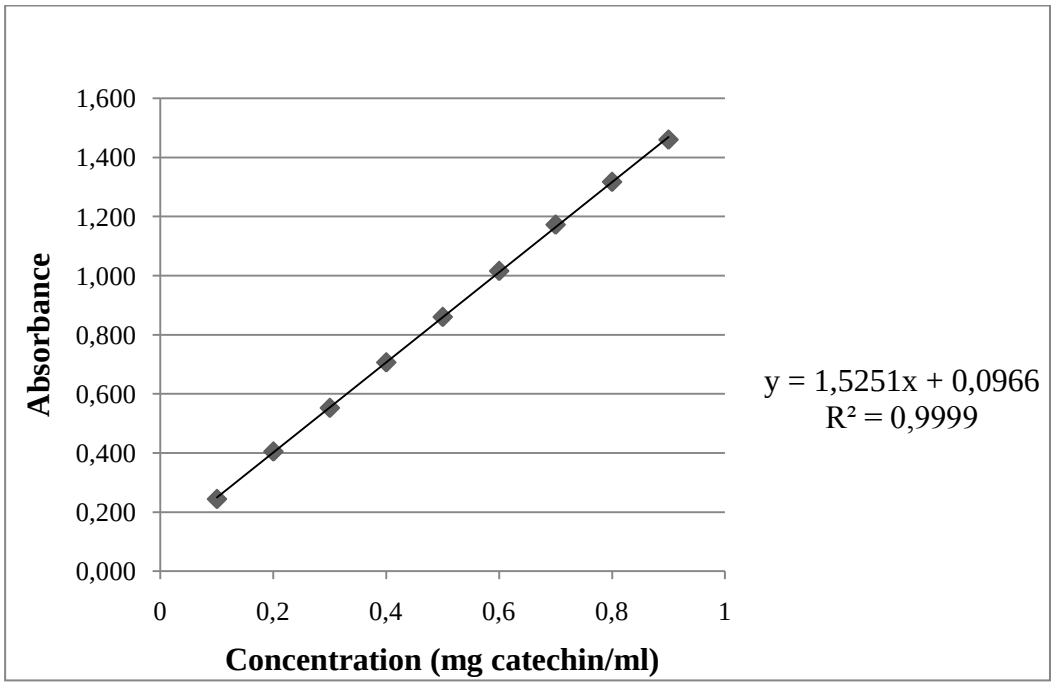

Figure C.1 : Catechin calibration curve for measuring total phenolic content

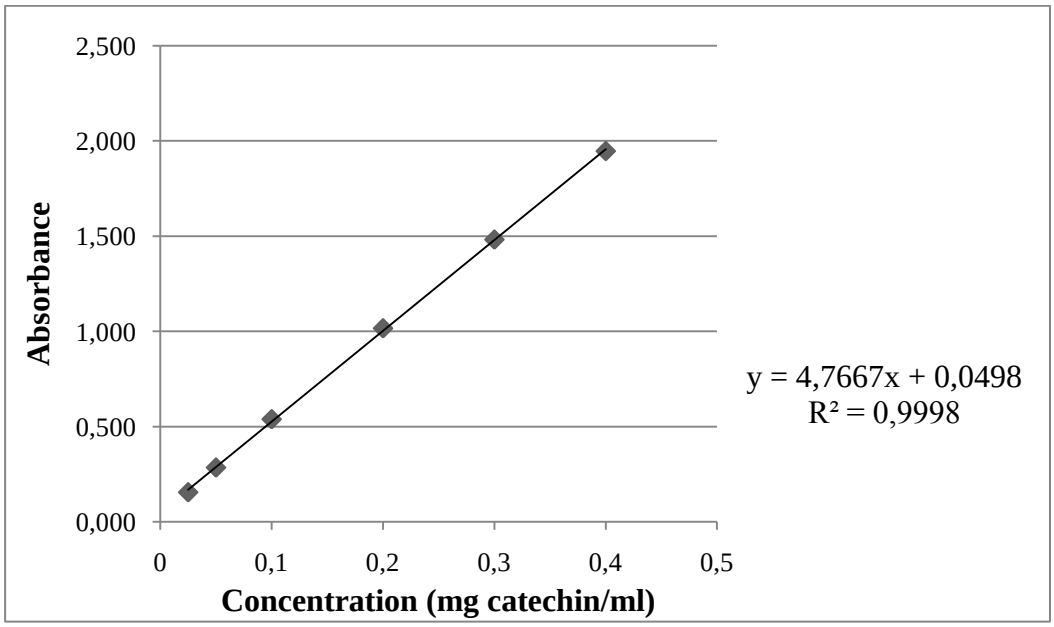

Figure C.2 : Catechin calibration curve for measuring total flavonoid content

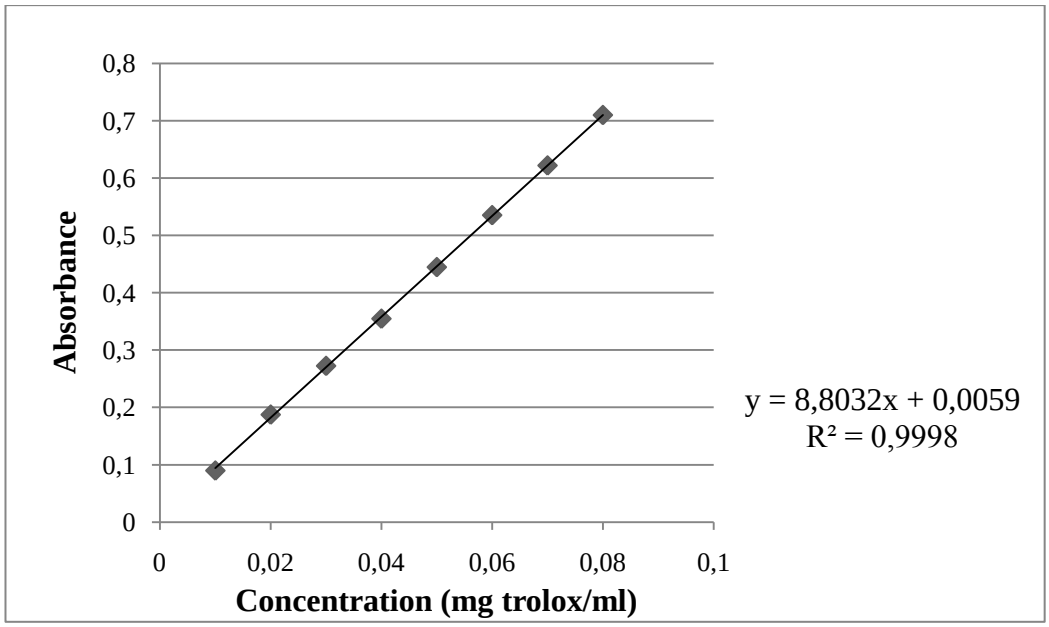

Figure C.3 : Trolox calibration curve for ABTS method

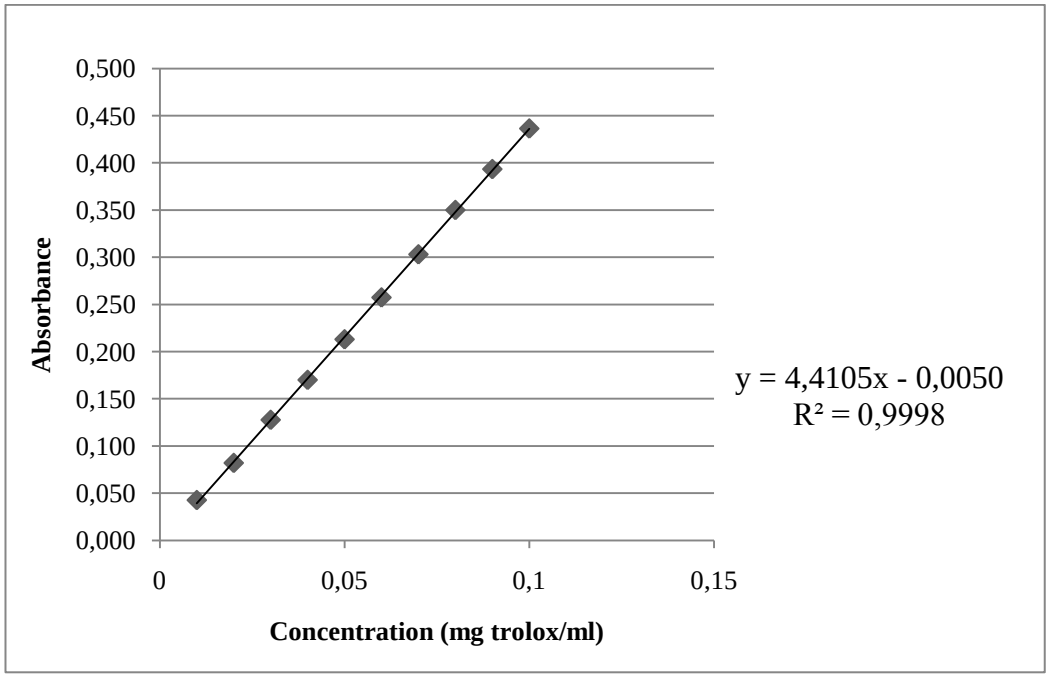

Figure C.4 : Trolox calibration curve for DPPH method

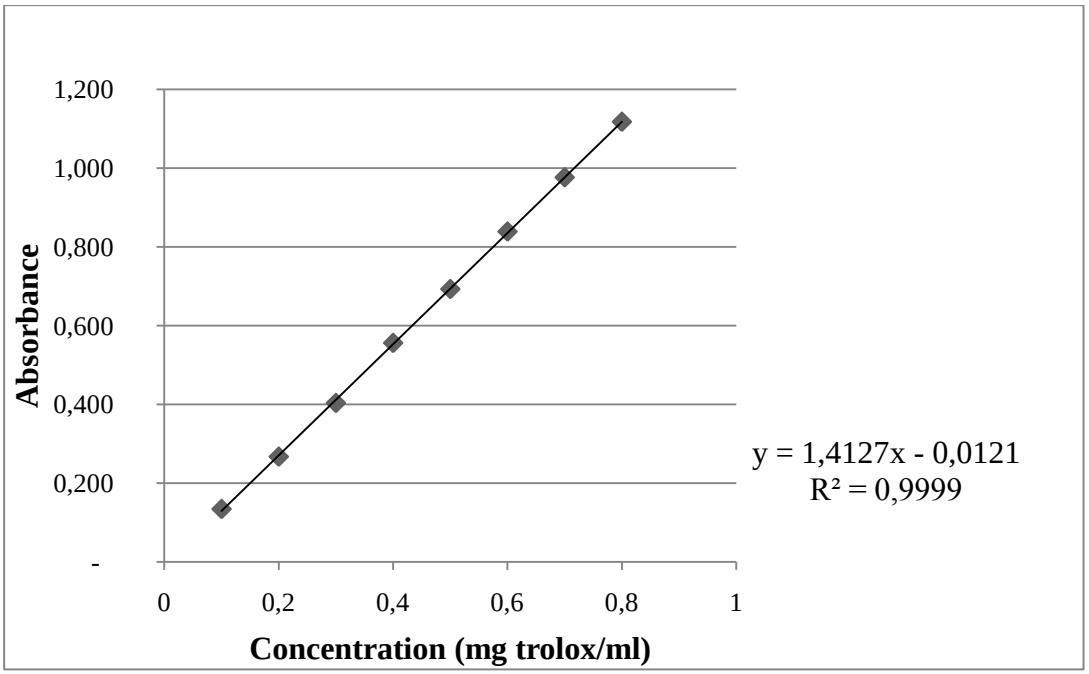

Figure C.5 : Trolox calibration curve for CUPRAC method

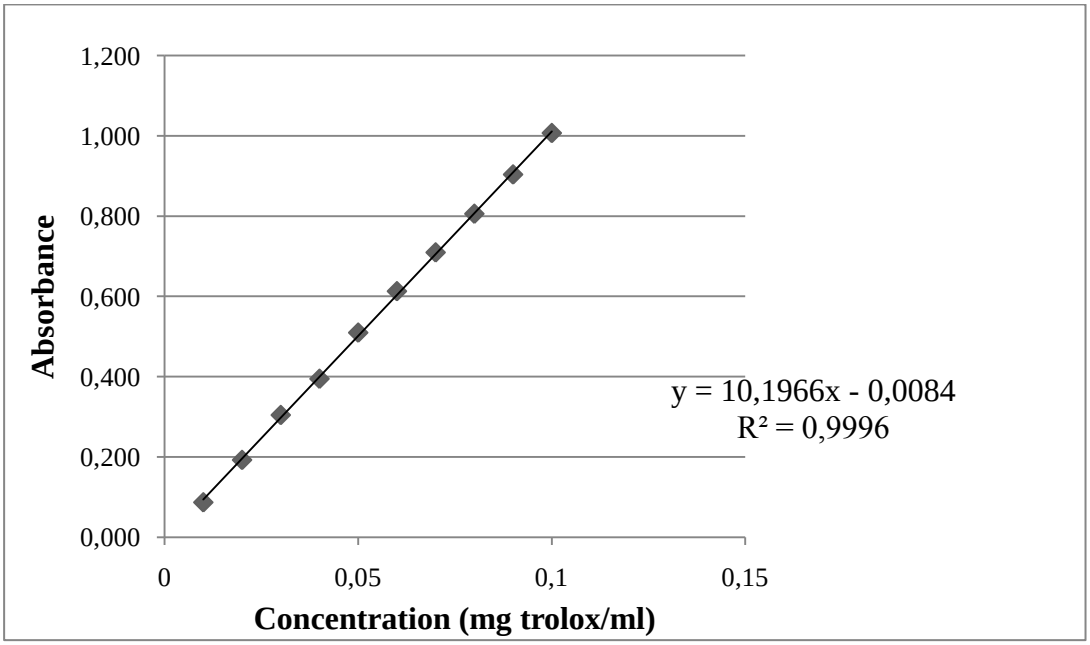

Figure C.6 : Trolox calibration curve for FRAP method

APPENDIX D : ANOVA Tables

Table D.1 : Results of ANOVA analysis applied on chocolate samples of mixing process

		Sum of Squares	df	Mean Square	F	Sig.
TOTAL PHENOLIC	Between Groups	212684,082	2	106342,041	503,224	0,000
	Within Groups	3169,821	15	211,321		
	Total	215853,903	17			
TOTAL FLAVONOID	Between Groups	56129,166	2	28064,583	238,943	0,000
	Within Groups	1761,796	15	117,453		
	Total	57890,962	17			
ABTS	Between Groups	5695876,847	2	2847938,423	64,889	0,000
	Within Groups	658345,017	15	43889,668		
	Total	6354221,863	17			
DPPH	Between Groups	4517707,279	2	2258853,640	221,266	0,000
	Within Groups	153131,824	15	10208,788		
	Total	4670839,103	17			
CUPRAC	Between Groups	28564365,809	2	14282182,904	551,274	0,000
	Within Groups	388613,674	15	25907,578		
	Total	28952979,483	17			
FRAP	Between Groups	4543051,312	2	2271525,656	126,991	0,000
	Within Groups	268309,764	15	17887,318		
	Total	4811361,076	17			

Table D.2 : Results of ANOVA analysis applied on chocolate samples of refining process

		Sum of Squares	df	Mean Square	F	Sig.
TOTAL PHENOLIC	Between Groups	96387,201	2	48193,601	154,406	0,000
	Within Groups	4681,830	15	312,122		
	Total	101069,031	17			
TOTAL FLAVONOID	Between Groups	136436,191	2	68218,096	269,170	0,000
	Within Groups	3801,578	15	253,439		
	Total	140237,769	17			
ABTS	Between Groups	7971204,372	2	3985602,186	57,607	0,000
	Within Groups	1037786,352	15	69185,757		
	Total	9008990,725	17			
DPPH	Between Groups	4166444,416	2	2083222,208	184,008	0,000
	Within Groups	169820,515	15	11321,368		
	Total	4336264,930	17			
CUPRAC	Between Groups	8689588,440	2	4344794,220	29,634	0,000
	Within Groups	2199196,997	15	146613,133		
	Total	10888785,438	17			
FRAP	Between Groups	1265886,874	2	632943,437	55,418	0,000
	Within Groups	171319,051	15	11421,270		
	Total	1437205,925	17			

Table D.3 : Results of ANOVA analysis applied on chocolate samples of conching process

		Sum of Squares	df	Mean Square	F	Sig.
TOTAL PHENOLIC	Between Groups	145142,333	2	72571,167	76,683	0,000
	Within Groups	14195,667	15	946,378		
	Total	159338,000	17			
TOTAL FLAVONOID	Between Groups	89619,111	2	44809,556	50,161	0,000
	Within Groups	13399,833	15	893,322		
	Total	103018,944	17			
ABTS	Between Groups	6467427,317	2	3233713,659	23,493	0,000
	Within Groups	2064703,992	15	137646,933		
	Total	8532131,309	17			
DPPH	Between Groups	7064942,595	2	3532471,298	131,031	0,000
	Within Groups	404385,893	15	26959,06		
	Total	7469328,488	17			
CUPRAC	Between Groups	32503698,977	2	16251849,49	75,136	0,000
	Within Groups	3244480,127	15	216298,675		
	Total	35748179,104	17			
FRAP	Between Groups	7574275,448	2	3787137,724	95,542	0,000
	Within Groups	594579,117	15	39638,608		
	Total	8168854,565	17			

Table D.4 : Results of ANOVA analysis applied on chocolate samples of tempering process

		Sum of Squares	df	Mean Square	F	Sig.
TOTAL PHENOLIC	Between Groups	113261,59	2	56630,795	120,882	0,000
	Within Groups	7027,201	15	468,480		
	Total	120288,791	17			
TOTAL FLAVONOID	Between Groups	24375,151	2	12187,576	35,870	0,000
	Within Groups	5096,490	15	339,766		
	Total	29471,642	17			
ABTS	Between Groups	5663342,682	2	2831671,341	85,806	0,000
	Within Groups	495010,010	15	33000,667		
	Total	6158352,691	17			
DPPH	Between Groups	4378617,733	2	2189308,866	142,523	0,000
	Within Groups	230416,223	15	15361,082		
	Total	4609033,956	17			
CUPRAC	Between Groups	6130709,846	2	3065354,923	24,289	0,000
	Within Groups	1893048,254	15	126203,217		
	Total	8023758,101	17			
FRAP	Between Groups	3605646,224	2	1802823,112	116,82	0,000
	Within Groups	231487,643	15	15432,510		
	Total	3837133,867	17			

Table D.5 : Results of ANOVA analysis applied on chocolate final products

		Sum of Squares	df	Mean Square	F	Sig,
TOTAL PHENOLIC	Between Groups	5097,709	2	2548,854	8,008	0,004
	Within Groups	4774,188	15	318,279		
	Total	9871,897	17			
TOTAL FLAVONOID	Between Groups	2910,034	2	1455,017	6,591	0,009
	Within Groups	3311,276	15	220,752		
	Total	6221,310	17			
ABTS	Between Groups	427500,430	2	213750,215	5,15	0,020
	Within Groups	622554,918	15	41503,661		
	Total	1050055,349	17			
DPPH	Between Groups	202547,361	2	101273,680	7,947	0,004
	Within Groups	191149,485	15	12743,299		
	Total	393696,846	17			
CUPRAC	Between Groups	940906,260	2	470453,130	9,374	0,002
	Within Groups	752828,548	15	50188,570		
	Total	1693734,807	17			
FRAP	Between Groups	322716,408	2	161358,204	7,548	0,005
	Within Groups	320677,270	15	21378,485		
	Total	643393,678	17			

Table D.6 : Results of ANOVA analysis applied on formulation A chocolate samples for all manufacturing processes

		Sum of Squares	df	Mean Square	F	Sig.
TOTAL PHENOLIC	Between Groups	139898,806	4	34974,702	87,207	0,000
	Within Groups	10026,378	25	401,055		
	Total	149925,184	29			
TOTAL FLAVONOID	Between Groups	174477,841	4	43619,460	128,836	0,000
	Within Groups	8464,172	25	338,567		
	Total	182942,013	29			
ABTS	Between Groups	19083554,156	4	4770888,539	83,870	0,000
	Within Groups	1422109,069	25	56884,363		
	Total	20505663,226	29			
DPPH	Between Groups	5574092,398	4	1393523,100	81,931	0,000
	Within Groups	425211,924	25	17008,477		
	Total	5999304,322	29			
CUPRAC	Between Groups	33202018,152	4	8300504,538	105,133	0,000
	Within Groups	1973810,628	25	78952,425		
	Total	35175828,780	29			
FRAP	Between Groups	7447926,862	4	1861981,715	90,942	0,000
	Within Groups	511861,538	25	20474,462		
	Total	7959788,400	29			

Table D.7 : Results of ANOVA analysis applied on formulation B chocolate samples for all manufacturing processes

		Sum of Squares	df	Mean Square	F	Sig.
TOTAL PHENOLIC	Between Groups	189497,101	4	47374,275	74,440	0,000
	Within Groups	15910,254	25	636,410		
	Total	205407,356	29			
TOTAL FLAVONOID	Between Groups	155621,333	4	38905,333	101,602	0,000
	Within Groups	9572,969	25	382,919		
	Total	165194,302	29			
ABTS	Between Groups	28415293,406	4	7103823,351	149,155	0,000
	Within Groups	1190676,386	25	47627,055		
	Total	29605969,792	29			
DPPH	Between Groups	4657716,173	4	1164429,043	67,960	0,000
	Within Groups	428348,993	25	17133,960		
	Total	5086065,166	29			
CUPRAC	Between Groups	33310797,844	4	8327699,461	66,862	0,000
	Within Groups	3113764,674	25	124550,587		
	Total	36424562,519	29			
FRAP	Between Groups	4347809,394	4	1086952,348	42,902	0,000
	Within Groups	633399,601	25	25335,984		
	Total	4981208,995	29			

Table D.8 : Results of ANOVA analysis applied on formulation C chocolate samples for all manufacturing processes

		Sum of Squares	df	Mean Square	F	Sig.
TOTAL PHENOLIC	Between Groups	390831,928	4	97707,982	308,731	0,000
	Within Groups	7912,075	25	316,483		
	Total	398744,003	29			
TOTAL FLAVONOID	Between Groups	524296,357	4	131074,089	351,073	0,000
	Within Groups	9333,833	25	373,353		
	Total	533630,190	29			
ABTS	Between Groups	30053353,644	4	7513338,411	82,906	0,000
	Within Groups	2265614,833	25	90624,593		
	Total	32318968,477	29			
DPPH	Between Groups	12642925,851	4	3160731,463	267,547	0,000
	Within Groups	295343,023	25	11813,721		
	Total	12938268,874	29			
CUPRAC	Between Groups	83248128,437	4	20812032,109	153,454	0,000
	Within Groups	3390592,298	25	135623,692		
	Total	86638720,736	29			
FRAP	Between Groups	8767081,732	4	2191770,433	124,219	0,000
	Within Groups	441111,707	25	17644,468		
	Total	9208193,440	29			

APPENDIX E: Sensory analysis panel form

Name & Surname : _____ **Test No :_____**
Sample No : _____ **Date : _____**

QUANTITATIVE DESCRIPTIVE ANALYSIS OF CHOCOLATE PANEL FORM

Please first smell and then taste the coded chocolate samples starting from left to right. Rate each of the descriptives of "odor", "taste and aroma" and "texture" , respectively using 0-7 category scale and mark the bestfit cell with "X". Zero represents the minimum intensity and the seven represents the maximum intensity.

No	Descriptives	ODOR : Descriptions	0	1	2	3	4	5	6	7
1	Cocoa Odor	This describes the intensity of cocoa odor without breaking the chocolate by smelling **(NONE:0-VERY MUCH:7)**								
2	Almond Odor	This describes the intensity of almond odor without breaking the chocolate by smelling **(NONE:0-VERY MUCH:7)**								
3	Vanillin Odor	This describes the intensity of vanillin odor without breaking the chocolate by smelling **(NONE:0-VERY MUCH:7)**								
No	**Descriptives**	**TASTE and AROMA : Descriptions**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
5	Cocoa Aroma	This describes the intensity of the cocoa aroma sensed while chewing the product by pushing the air in the oral cavity (mouth is closed) to the nasal cavity **(NONE:0-VERY MUCH:7)**								
6	Vanillin Aroma	This describes the intensity of the vanillin aroma sensed while chewing the product by pushing the air in the oral cavity (mouth is closed) to the nasal cavity **(NONE:0-VERY MUCH:7)**								
7	Almond Aroma	This describes the intensity of the almond aroma sensed while chewing the product by pushing the air in the oral cavity (mouth is closed) to the nasal cavity **(NONE:0-VERY MUCH:7)**								
8	Alkaline Taste	This describes the intensity of the burning sensation at the front of tongue when the product is at first taken to mouth **(NONE:0-VERY MUCH:7)**								
9	Sweetness	This describes the intensity of the sweet taste after the product is melted into mouth **(NONE:0-VERY MUCH:7)**								
10	Bitter Taste	This describes the intensity of the bitter taste after the product is melted into mouth **(NONE:0-VERY MUCH:7)**								
11	Oily Taste	This describes the intensity of the oily taste after the product is melted into mouth **(NONE:0-VERY MUCH:7)**								
12	Earth Taste	This describes the intensity of the earth taste after the product is swallowed **(NONE:0-VERY MUCH:7)**								
No	**Descriptives**	**TEXTURE : Descriptives**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	Melting rate	The level of chocolate melting in the hand **(NONE:0 – VERY MUCH:7)**								
2	Firmness	This is the measure of the amount of force needed to bite through a piece of chocolate by using incissors **(VERY SOFT:0-VERY HARD:7)**								
3	Brittleness	This describes the level of easiness of the spreading of the product into mouth while chewing with grinders **(VERY EASY:0-VERY DIFFICULT:7)**								
4	Slippery	This describes the level of easiness of the product slipping on the tongue while it is melting **(VERY EASY:0-VERY DIFFICULT:7)**								
5	Adhesiveness	This is the measure of the sticked product to grinders while chewing **(NONE:0-VERY MUCH:7)**								
6	Oiliness	This is the measure of the amount of oil that surrounds the mouth while chewing **(NONE:0-VERY MUCH:7)**								
7	Water absorption	This is the amount of decrease of saliva in the mouth while chewing **(NONE:0-VERY MUCH DECREASE:7)**								
8	Disappearance in mouth	This is the length of elapsed time that is needed to swallow a product with the help of teeth, tongue, palate and saliva after it is taken into mouth **(VERY SLOW:0 – VERY FAST:7)**								
9	Puckery mouthfeel	The level of puckering sensation characterized along the sides of tongue after the product is swallowed **(NONE:0 - VERY MUCH:7)**								

Figure E.1 : Panel form of quantitative descriptive analysis of chocolate

APPENDIX F: T-Test Table

Table F 1 : T-test analysis to compare the total phenolic and flavonoid contents and antioxidant capacities of ingredients used in the first and the second production

FirstProduction vs SecondProduction		Paired Differences							
					95% Confidence Interval of the Difference				
		Mean	Std. Deviation	Std. Error Mean	Lower	Upper	t	df	Sig. (2-tailed)
DEKINNED ALMOND PASTE	ABTS	8,951	13,819	5,641	-5,551	23,453	1,587	5	,173
	CUPRAC	34,879	9,062	3,699	25,370	44,389	9,428	5	,000
	DPPH	14,166	5,256	2,146	8,650	19,682	6,602	5	,001
	TOTAL FLAVONOIDS	,273	1,530	,624	-1,332	1,878	,438	5	,680
	FRAP	10,906	1,411	,576	9,425	12,386	18,933	5	,000
	TOTAL PHENOLICS	,962	,553	,226	,381	1,542	4,261	5	,008
ALKALIZED CACAO POWDER	ABTS	-182,375	169,660	56,553	-312,787	-51,963	-3,225	8	,012
	CUPRAC	-804,427	220,476	73,492	-973,900	-634,954	-10,946	8	,000
	DPPH	-515,658	65,817	21,939	-566,249	-465,066	-23,504	8	,000
	TOTAL FLAVONOIDS	-68,848	9,586	3,195	-76,217	-61,479	-21,546	8	,000
	FRAP	-252,940	39,431	13,144	-283,249	-222,630	-19,244	8	,000
	TOTAL PHENOLICS	-44,289	8,437	2,812	-50,774	-37,804	-15,748	8	,000
CACAO MASS	ABTS	1958,217	563,455	187,818	1525,107	2391,326	10,426	8	,000
	CUPRAC	2680,376	1873,407	624,469	1240,348	4120,404	4,292	8	,003
	DPPH	1585,219	1224,676	408,225	643,850	2526,589	3,883	8	,005
	TOTAL FLAVONOIDS	215,651	137,645	45,882	109,847	321,454	4,700	8	,002
	FRAP	1497,613	1096,000	365,333	655,153	2340,073	4,099	8	,003
	TOTAL PHENOLICS	202,330	138,767	46,256	95,664	308,995	4,374	8	,002

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