ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INVESTIGATING THE MATRIX EFFECT OF BLUEBERRY, OAT MEAL AND MILK ON POLYPHENOLS, ANTIOXIDANT ACTIVITY AND POTENTIAL BIOAVAILABILITY

M.Sc. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

MAVİYEMİŞ, YULAF EZMESİ VE SÜTÜN MATRİKS ETKİSİNİN POLİFENOLİKLER, ANTİOKSİDAN AKTİVİTE VE POTANSİYEL BİYOYARARLILIK ÜZERİNDEKİ ETKİSİNİN İNCELENMESİ

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FOREWORD

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TABLE OF CONTENTS

Page

ABBREVIATIONS

ABTS : 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid
ANOVA : Analysis of variance
AOX : Antioxidant
C3GE : Cyanidin-3-glucoside equivalent
CE : Catechin equivalent
CUPRAC : Cupric ion reducing antioxidant capacity
DPPH : 2,2- diphenyl- picrylhydrazyl
ET : Electron transfer
FRAP : Ferric ion reducing antioxidant power
GAE : Gallic acid equivalent
HAT : Hydrogen atom transfer
HPLC : High performance liquid chromatography
PG : Postgastric
SD : Standart deviation
TAA : Total antioxidant activity
TAC : Total anthocyanin content
TEAC : Trolox equivalent antioxidant capacity
TFC : Total flavonoid content
TPC : Total phenolic content
UV : Ultraviole

LIST OF TABLES

Page

LIST OF FIGURES

Page

INVESTIGATING THE MATRIX EFFECT OF BLUEBERRY, OAT MEAL AND MILK ON POLYPHENOLS, ANTIOXIDANT ACTIVITY AND POTENTIAL BIOAVAILABILITY

SUMMARY

In recent years, berries have gained growing consumers' attention due to their potential health benefits. Among berries, blueberries have been known for their anticarcinogenic effects due to their ability to reduce oxidative stress and remedy the negative effects of oxidative stress. These properties made blueberries popular and many studies were carried out about them.

Furthermore, dietary fiber rich foods such as oat has gained consumers' attention. Although people prefer to consume ready to eat foods, they also demand health promoting effects from foods. As a result, food manufacturers are expected to produce healthful products by providing not only dietary fiber ingredient but also polyphenolic content with added fruit ingredients in formulations of ready-to eat breakfat cereals. These products are generally consumed with whole or skimmed milk. However, there was no available data or information in literature about the the effect of milk addition on the phenolic profile of breakfast cereals.

The aim of this study to investigate the matrix effect of blueberry, oat meal and milk on antioxidative potential, total phenolic, flavonoid and anthocyanin contents as well as their potential bioavailability.

In this study, ingredients of breakfast cereals and and combinations of the ingredients were investigated for their phenolic properties, antioxidative activity and bioaccessibility. One variety of blueberry (*Vaccinium arctostaphylos L.)* from northeastern region of Turkey was used for the preparation of mixtures. Blueberry, whole milk or skimmed milk and oat meal were blended together at specific ratios [(4:1:8) for oat meal/blueberry/milk; w/w/w] in order to prepare a breakfeast cereal that's similar to consumers' habits for consumption. Total phenolic, total flavonoid, total anthocyanin content analyses were performed for samples and total antioxidant capacity were estimated by using FRAP (Ferric reducing ability of plasma), DPPH (2,2- diphenyl-1-picrylhydrazyl), CUPRAC (Cupric ion reducing antioxidant capacity) and ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] assays. In addition, bioaccessibility of samples were analyzed by using in vitro digestion method.

Total phenolic content of blueberry *(Vaccinium arctostaphylos L.)* was found to be 12.43±0.34 mg gallic acid equivalent (GAE)/g fresh weight (fw). Oat meal, whole milk and skimmed milk were found to have 1.03±0.04, 0.53 ±0.02 and 0.50±0.01 mg GAE /g fresh weight, respectively. The flavonoid content of the samples were 4.67 ±0.12; 0.36 ±0.03; 0.22 ±0.01; 0.18 ±0.00 mg catechin equivalent/g fresh weight (mg catechin equivalent (CE)/g fw) for blueberry, oat meal, whole milk and skimmed milk samples, respectively. Oat meal sample and both milk samples were found to contain no anthocyanin content. No anthocyanin was detected. On the other hand,

blueberry sample was found to be a rich anthocyanin source having 2.51±0.04 mg cyanidin 3-glucoside equivalent (C3GE)/g fresh weight. Among the antioxidant capacity assays, CUPRAC method was concluded with the highest antioxidant capacity for all of the samples.

The results from combination of oat meal, whole milk or skimmed milk (OM1, OM2) revealed that whole milk had an inhibition effect on total phenolic and flavonoid contents. However, skimmed milk had only an additive effect just like expected on these values. Other combinations except for OB (oat meal+blueberry) resulted in an inhibiton effect due to whole and skimmed milk addition. Skimmed milk was found to have higher inhibiton rate than whole milk on the samples. On the other hand, antioxidant capacity assay results showed variations among methods.

According to HPLC analysis, phenolic acids such as caffeic, ferulic and gallic acid were found in oat meal samples and chlorogenic, p-coumaric acid were found in blueberry samples. Catechin was not detected in blueberry samples but was detected in oat samples. The detected flavonols in the blueberry samples were quercetin-3-galactoside and quercetin-3-ß-d-glucoside. Cyanidin chloride, cyanidin 3-o glucoside, cyanidin-3-o-rutinoside chloride, delphinidine chloride, delphinidin 3-glucoside, malvidin-3-galactoside, pelargonin chloride, pelargonidin-3-O-glucoside, peonidin-3-glucoside and petunidin chloride were the detected anthocyanins in blueberry sample and their contents were 0.69±0.00, 7.59±0.54, 4.03±1.25, 18.28±0.00, 30.61±1.64, 5.4±0.2, 68.53±4.6, 16.20±0.00, 1.41±0.00 and 7.00±0.00 mg/100 g fw, respectively.

Combinations of oat meal, blueberry, whole milk and skimmed milk were also analyzed for their phenolic profile by HPLC. Although catechin had been detected in oat samples, it was not found in OM1 (oat meal+whole milk) and OM2 (oat meal+skimmed milk) samples, pointing a potential interaction between catechin and milk proteins as also reported in the literature previously. Many of the phenolics could not be detected in BM1 (blueberry+whole milk) and BM2 (blueberry+skimmed milk) samples due to dilution of samples or potential interactions between phenolics and milk proteins. The results from OBM1 (oat meal+blueberry+whole milk) and OBM2 (oat meal+blueberry+skimmed milk) samples were based on OB HPLC results and effect of milk was investigated due to milk addition on OB sample. Catechin, caffeic acid and *p*-cuomaric acid were not detected in OBM1 and OBM2 samples. The anthocyanins showed lower contents than expected values except for malvidin-3-galactoside, due to milk addition.

In vitro digestion procedure was applied to samples in order to assess the potential bioavailability of samples. Postgastric (PG), IN (partition inside dialysis tube) and OUT (partition outside dialysis tube) fractions of samples were analyzed by total phenolic content assay and the results were compared with total phenolic content results of acetone extracts of samples. The results showed that the recovery of phenolics from blueberry sample was low (53% for sum of IN and OUT samples). Oat meal was found to have higher recovery values than blueberry. Whole milk and skimmed milk had a higher total phenolic content recovery in IN sample compared to oat meal and blueberry. Moreover, they showed a higher total phenolic content recovery in PG, OUT than control samples after bioaccessibility assay. HPLC results showed that whole milk and skimmed milk had no phenolic acids.

Contrary to results of effect of milk on total phenolic content as discussed before, milk had a varied effect on total phenolic content of combined samples after

bioaccessibility assay. Milk addition had no effect on the potential bioavailability of PG and IN fractions from blueberry samples. However, an inhibiton effect was found on OUT fraction due to milk addition. Whole milk addition on oat meal and oat meal+blueberry sample had no significant effect for PG, IN and OUT fractions. On the other hand, skimmed milk caused a significant decrease in the total phenolic content of PG fraction, additive effect in the total phenolic content of IN fraction and synergistic effect in the total phenolic content of OUT fraction from OM2 sample.

According to total phenolic content results after bioaccessibility assay, milk addition did not affect the total phenolic content of IN partitions of samples significantly. Furthermore, milk addition on berry fruit will cause no significant influence on the bioacccessible partition from stomach so that consuming blueberry with milk do not damage total phenolic content. Whole milk addition on oat meal had no significant effect for PG, IN and OUT samples. Moreover, codigestion of oat meal with skimmed milk (like in the consumption of breakfast cereals) had no significant effect on bioaccessible amount from small intestine but synergistic effect was found on the total phenolic content of partition passing to large intestine.

HPLC profile of PG, IN and OUT partitions of samples were analyzed and revealed that gastric and intestinal conditions affected the stability of the phenolics. Results showed that gastric and intestinal conditions had negative effects on phenolic acid profile of samples. Most of the anthocyanins in blueberry were stable in gastric conditions, whereas increase in the amount of anthocyanins were found as a result of gastric digestion. However, anthocyanins were not stable in intestinal conditions. Based on our findings, anthocyanins were not detected in IN and OUT partitions of B, BM1 and BM2, OB, OBM1 and OBM2 samples. In addition, the stability of anthocyanins of BM1, BM2, OB, OBM1 and OBM2 were low during gastric digestion.

MAVİYEMİŞ, YULAF EZMESİ VE SÜTÜN MATRİKS ETKİSİNİN POLİFENOLİKLER, ANTİOKSİDAN AKTİVİTE VE POTANSİYEL BİYOYARARLILIK ÜZERİNDEKİ ETKİSİNİN İNCELENMESİ

ÖZET

Son yıllarda, polifenolik içeriği yüksek olan üzümsü meyveler sağlık üzerine olan olumlu etkileri sayesinde tüketicilerin dikkatini çekmiştir. Bu üzümsü meyveler arasında maviyemişler (*Vaccinium)*, oksidatif stresi azaltan ve oksidatif stresin olumsuz etkilerini iyileştiren ve dolayısıyla kanser önleyici özellikleri ile bilinmektedirler. Bu özellikleri maviyemişleri bilinen bir meyve haline getirmiş ve bu ürünler üzerine pek çok çalışmanın yapılması sağlamıştır. Türkiye'de maviyemişler üzerine yapılmış çalışmalar bulunmakla birlikte genel olarak yapılan çalışmalar farklı bölgelerde yetişen maviyemiş türlerinin fenolik profilini ortaya koymuştur.

Bir başka konu, yulaf gibi diyet lifi açısından zengin gıdaların sağlık üzerine olumlu etkilerinin dikkat çekmesidir. Günümüzde insanlar hazır gıdaları tercih etse de, bu gıdaların sağlık üzerine olumlu etkileri olmasını da talep etmektedir. Sonuç olarak üreticilerden ürettikleri ürünler ile tüketicilerin bu taleplerini karşılamaları beklenmektedir. Kahvaltılık gevrekler, hem diyet lifi içeriği hem de ürüne eklenen meyvelerden gelen fenolik madde içerikleri ile bu ürünlere iyi birer örnek teşkil etmektedir. Bu ürünler, yağlı veya yağsız sütle beraber tüketilmektedir ancak literatürde süt ilavesinin bu ürünlerin fenolik madde içeriği üzerine etkisini inceleyen çalışma bulunmamaktadır. Bazı kaynaklarda, süt ilavesinin çay, süt vb. gıda ürünlerinin antioksidan kapasitesine olumsuz etkileri rapor edilmişken, bazı kaynaklar sütün önemli bir etkisi olmadığını belirtmiştir. Bu çalışma, bu noktada süt ilavesinin meyveli ya da meyvesiz yulaflı kahvaltılık karışımların toplam fenolik, flavonoid ve antosiyanin içeriği ve potansiyel biyoyararlılığı üzerine etkisini inceleyebilmek için yapılmıştır.

Çalışmada polifenolik özelliklerini ortaya koyabilmek için yulaf bazlı kahvaltılık karışımları içeren örneklerin ingrediyenleri tek başına ve karışımlar halinde incelenmiştir. Doğu Karadeniz Bölgesinden temin edilen tek tür maviyemiş örneği kullanılmıştır. Maviyemiş, yağlı ya da yağsız süt ve yulaf gevreği ağırlıkça belirli oranlarda karıştırılarak tüketicinin tüketim alışkanlıklarını karşılayacak biçimde karışımlar hazırlanmıştır [(4:1:8) yulaf ezmesi/maviyemiş/süt]. Örneklere toplam fenolik, flavonoid ve antosiyanin analizi uygulanmış, antioksidan kapasite FRAP (Ferrik iyonu indirgeme kapasitesi), DPPH (2,2- diphenyl-1-picrylhydrazyl), CUPRAC (Bakır indirgeme gücü) and ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sülfonik asit)] metodlarıyla belirlenmiştir. Sonuçlar toplam fenolik içerik için gallik asit eşedeğeri, flavonoid içerik için kateşin eşedeğeri, antosiyanin içerik için siyanidin-3-glukozit eşdeğeri cinsinden ifade edilmiştir. Tüm antioksidan kapasite metodlarının sonuçları troloks eşdeğeri olarak belirtilmiştir. Ayrıca, örneklerin potansiyel biyoyararlılıklarının tespiti için in vitro sindirim yöntemi kullanılmıştır.

Örneklerin HPLC profillerinin belirlenmesi için literatürde kullanımı daha yaygın olan metanol ekstraksiyon çözgeni olarak kullanılmıştır. Analizler sonucunda maviyemişin toplam fenolik içeriği 12,43±0,34 mg gallik asit eşdeğeri (GAE)/g taze örnek bulunmuştur. Yulaf ezmesi,yağlı ve yağsız sütün toplam fenolik madde içerikleri sırasıyla 1,03±0,04, 0,53 ±0,02 and 0,50±0,01 mg GAE/g taze örnek bulunmuştur. Toplam flavonoid madde için 4,67 ±0.12; 0,36 ±0,03; 0,22 ±0,01; 0,18 ±0,00 mg kateşin eşdeğeri (CE)/g taze örnek sırasıyla maviyemiş, yulaf gevreği, yağlı ve yağsız sütte tespit edilen değerler olmuştur. Yulaf gevreği ve süt örneklerinde antosiyanin madde tespit edilmemiş, ancak maviyemiş içermiş olduğu 2,51±0,04 mg siyanidin 3-glukozit eşdeğeri (C3GE)/g taze örnek antosiyanin içeriği ile zengin bir antosiyanin kaynağı olduğunu doğrulamıştır. Uygulanan farklı antioksidan kapasite metodları örnekler için farklı sonuçlar vermiş ve tüm örnekler için en yüksek antioksidan kapasite CUPRAC metodunda saptanmıştır.

Yulaf ezmesi, yağlı veya yağsız süt karışımlarının (OM1,OM2) analiz sonuçlarına göre, yağlı süt toplam fenolik ve toplam flavonoid madde miktarı üzerinde inhibisyon etkisine neden olmakta iken yağsız sütün ise istatistiksel açıdan önemli bir etkisinin olmadığı görülmüştür. Diğer sütlü karışımlarda hem yağlı hem yağsız süt ilavesi örneklerin toplam fenolik, flavonoid ve antosiyanin madde içeriklerini istatistiksel açıdan önemli derecede düşürmüştür. Yağsız süt yağlı süte göre örneklerde daha yüksek oranlarda inhibisyon etkisi göstermiştir. Diğer yandan, antioksidan kapasite analiz metodlarından elde edilen sonuçlar metottan metoda farklılıklar göstermiştir.

Fenolik asit, flavonoid ve antosiyanin profilini belirlemek için örneklere HPLC analizi uygulanmıştır. Yulaf gevreği örneklerinde fenolik asitlerden başlıca kafeik, ferulik ve gallik asit tespit edilirken, maviyemiş örneklerinde klorojenik asit ve p-kumarik asit tespit edilmiştir. Maviyemiş örneklerinde tespit edilememesine rağmen yulaf örneklerinde kateşin tespit edilmiştir. Kuersetin-3-galaktozit ve kuersetin-3-ß-d-glukozit maviyemiş örneklerinde tespit edilen flavonollerdir. Maviyemiş örneklerinde antosiyanin olarak, siyanidin klorit, siyanidin 3-o glukozit, siyanidin 3-o-rutinozit, delfinidin klorit, delfinidin 3-glukozit, malvidin-3-galaktozit, pelargonin klorit, pelargonidin-3-o-glukozit, peonidin-3-glukozit and petunidin klorit tespit edilmiştir, tespit edilen miktarlar sırasıyla, 0,69±0,00, 7,59±0,54, 4,03±1,25, 18,28±0,00, 30,61±1,64, 5,40±0,20, 68,53±4,60, 16,20±0,00, 1,41±0,00 ve 7,00±0,00 mg/ 100 g taze örnek ağırlığıdır.

Yulaf gevreği, maviyemiş, yağlı ve yağsız süt örneklerinin karışımlarının HPLC profilleri de incelenmiştir. Yulaf örneklerinde tespit edilmesine rağmen, yulaf-süt karışımı örneklerinde (OM1, OM2) kateşin tespit edilmemiştir, bu durum literatürde belirtilen kateşin ile süt proteinleri arasında gerçekleşebilecek potansiyel bir etkileşime işaret etmektedir. Pek çok fenolik bileşik igrediyenlerin fenolik profilinde tespit edilmesine rağmen karışımlarda tespit edilememiştir. OBM1 ve OBM2 örneklerinde sütün etkisi incelenirken OB örneği temel alınmış olup, OB üzerine süt ilavesinin etkisi tartışılmıştır. OBM1 ve OBM2 örneklerinde kateşin, kafeik asit ve *p*-kumarik asit tespit edilmemiştir. Malvidin-3-galaktozit dışındaki antosiyaninler süt ilavesi sonucu beklenen değerin altında bulunmuştur.

Örneklerin potansiyel biyoyararlığını ifade eden biyoerişilebilirliğini saptayabilmek için in vitro sindirim yöntemi uygulanmıştır. Örneklerden in vitro sindirim prosedürü sonucunda elde edilen PG (postgastrik, midedeki sindirim prosedürü sonrası diyaliz tüpünde kalan kısım), IN (bağırsakta sindirim prosedürü sonrası diyaliz tüpüne

geçen) ve OUT (bağırsaktaki sindirim prosedürü sonrası diyaliz tüpüne geçmeyen) kısımlarına toplam fenolik madde analizi uygulanmış ve sonuçlar örneklerin aseton ekstraktlarının fenolik içerik sonuçlarıyla karşılaştırılmıştır. Maviyemiş örneklerden in vitro sindirim sonucu fenolik içeriğin kazanımı düşük çıkmıştır. IN ve OUT fraksiyonlarının toplam fenolik içerikleri kontrol değerinin %53'ü olarak bulunmuştur. Bu değer yulaf ezmesinde daha yüksek bulunmuştur. Yağlı ve yağsız sütün ise in vitro sindirim sonucunda, toplam fenolik madde analizinde maviyemiş ve yulaf ezmesine göre çok daha yüksek değerler tespit edilmiştir. Üstelik, süt örneklerinin PG ve OUT fraksiyonlarının toplam fenolik madde analizi sonucu elde edilen değerler kontrolden daha yüksek çıkmıştır. HPLC sonuçları, tahminlendiği üzere yağlı ve yağsız sütte fenolik içerik olmadığını tespit etmiştir, toplam fenolik içerik tayinindeki pozitif sonuçlar süt proteinlerinden kaynaklanmaktadır.

İn vitro sindirim sonucunda süt ilavesinin karışımlarda farklı etkiler gösterdiği belirlenmiştir. Örneğin, maviyemiş örneklerinin PG ve IN fraksiyonları üzerinde yağlı ya da yağsız süt ilavesinin önemli bir etkisi olmadığı tespit edilmiş, ancak OUT fraksiyonunda yağlı ve yağsız süt ilavesi toplam fenolik içerik üzerine inhibisyon etkisi göstermiştir. Yulaf ezmesi (O) ve yulaf ezmesi+maviyemiş örneğine (OB) yağlı süt ilavesi PG, IN ve OUT fraksiyonlarında toplam fenolik madde içeriğini etkilememiştir. Diğer taraftan, yağsız süt ilavesi, OM2 örneğinin toplam fenolik madde içeriği üzerinde PG fraksiyonunda inhibisyon, OUT fraksiyonunda sinerjistik etki göstermiş, IN fraksiyonunda önemli bir değişime neden olmamıştır.

İn vitro sindirim sonuçlarına göre, yağlı veya yağsız süt ilavesi örneklerin IN fraksiyonlarının toplam fenolik madde içeriğini etkilememiştir. Ayrıca, maviyemiş üzerine yağlı, yağsız süt ilavesi PG fraksiyonu üzerine de etki göstermemiştir, böylece maviyemiş üzerine yağlı ya da yağsız süt ilavesinin in vitro sindirim sayesinde toplam fenolik madde içeriğe olumsuz bir etkisi gözlenmemiştir. Maviyemiş+yulaf gevreği karışımlarının sütle karışımlarının in vitro sindirim sonucunda elde edilen PG, IN ve OUT fraksiyonu sonuçlarına göre meyve içeren kahvaltılık gevreklerin yağlı veya yağsız sütle karıştırılması fenolik madde içerikleri üzerine olumsuz etki göstermemiştir.

PG, IN ve OUT kısımlarının HPLC profili incelendiğinde, mide ve bağırsak koşullarının fenoliklerin stabilitesini etkilediği görülmüştür. Sonuçlara göre, mide ve bağırsak koşulları örneklerin fenolik asit profili üzerine olumsuz etki göstermiştir. Antosiyaninlerin çoğunluğu mide koşullarında stabilitesini koruyabilmiştir. Buna rağmen, bağırsak koşullarında stabil kalamamışlardır. Sonuç olarak B, BM1, BM2, OB, OBM1 ve OBM2 örneklerinin IN ve OUT kısımlarında antosiyanin tespit edilememiştir. Üstelik BM1, BM2, OB, OBM1 ve OBM2 karışımlarındaki antosiyaninlerin midedeki sindirim prosedürüne dayanıklılığı da düşük bulunmuştur.

Çalışma, örneklerin in vitro sindirim yöntemiyle belirlenen biyoerişilebilirliğinden yola çıkarak potansiyel biyoyararlıklarını tespit etmiştir. Biyoyararlığın gerçek manada tespiti için incelenen polifenolik bileşiklerin absorpsiyonunun yanısıra vücuttaki aktivitesinin de değerlendirilmesi gerekmektedir ancak bu çalışmaların yürütülmesi zor ve son derece zaman alıcıdır. Bu nedenle çalışma potansiyel biyoyararlığın in vitro sindirim metoduyla tespiti yöntemiyle yürütülmüştür.

1.INTRODUCTION

Oxidative stress can be explained by the breakdown of the balance between oxido-reductive reactions and excessive production of reactive oxygen species (Poljack-Blazi *et al*., 2010). The reasons of oxidative stress can be sourced from decreased levels of antioxidants or increased production of reactive species (Halliwell, 2001).

Polyphenols can represent antioxidant properties owing to their redox potential. They are able to react as reducing agents, hydrogen donors, metal chelators and singlet oxygen scavengers so that they have gained much more attention (Rice- Evans *et al*., 1996).

Blueberry (*Vaccinium arctostaphylos L.*) is an important fruit due to its high polifenolic, anthocyanin content and as well as its potential health benefits. Blueberries were reported to possess anticancer effects owing to their oxidative stress reducing ability. Blueberries were known as serious phytochemical sources and these phytochemicals also block treatment resisting pathways of tumor cells (Seeram, 2006).

In recent years, dietary fiber rich foods such as oat attracted the attention of consumers in terms of well-balanced and healthy diets. Oats have been consumed by humans since ancient times and accepted as a healthy food so that it has gained consumers' attention and consumption of oat and oat-derived products has showed a growing trend due to the results of the researches reporting its hypocholesterolemic and hypoglycemic effects (Mälkki, 2001).

Ready-to-eat breakfast cereals together with health promoting foods such as berries have gained attention as a result of their health beneficial effects. These subjects have made it available to find oat-based breakfast cereals with dried fruit contents. These ready-to-eat breakfast cereals are consumed with either skimmed or whole fat milk at different ratios due to consumers' preference. However, there is no available information about changes in antioxidant potential and bioavailability of these ingredients when consumed together.

Many foods such as coffee, tea, breakfast cereals, infant cereals are consumed with milk. There are researches available which investigated the effect of whole milk, skimmed milk or semi-skimmed milk on antioxidant capacity of tea, coffee, chocolate and blueberry when together consumed with milk. However, many of the studies represented contradictory results. Some of the studies reported the inhibition effect of milk whereas other studies represented no significant effect of milk (Langley-Evans, 2000; Sharma *et al*., 2008; Serafini *et al*., 2009; Reddy *et al*., 2005; Dupas *et al*., 2006a).

Another important issue for polyphenols are their bioactivity as being an effective nutritional ingredient. Many studies have been conducted to assess the bioavailability of phenolics in vivo or in vitro. ”Bioavailability” term consists of several sequential steps including the availability for absorption, metabolism, tissue distribution, and bioactivity. However, “bioaccessibility” term refers to the proportion that is absorbed from intestinal tract (Fernández-García *et al*, 2009). In vitro digestion process was used in our study to determine the bioaccessibility of the samples, it will indicate the potential bioavailability of the samples.

This study was designed to investigate the matrix effects of blueberry, oat meal and milk on antioxidative potential, total phenolic, flavonoid and anthocyanin contents as well as their potential bioavailability. In addition, skimmed milk and whole milk were used to prepare the mixtures to determine the effect of fat content in milk. The aim of the study was also to give recommendations to the consumers for best consumption practices in terms of higher antioxidative benefit and bioavailability.

2. LITERATURE REVIEW

2.1 Antioxidants and Free Radicals

Oxygen is an essential part of life for many living organims to survive. However, it is also susceptible of having negative effects on living organisms because of causing formation of free radicals (FR) and reactive species (RS) (Boskou and Elmadfa, 2010). A molecular entity that have at least one unpaired electron is defined as "free radical" (Madhavi *et al*., 1996). Active oxygen species donate oxygen including molecules and some of them are called free radicals due to possessing unpaired electrons. Examples to active oxygen species are shown in Table 2.1.

Table 2.1: Active oxygen and related species (Jadhav *et al*., 1996).

Radicals		**Non-radicals**	
O_2^{*-}	superoxide	H_2O_2	hydrogen peroxide
HO^*	hydroxyl radical	$^1O_2$	singlet oxygen
HO_2^*	hydroperoxyl radical	LOOH	lipid hydroperoxide
L^*	lipid radical	Fe=O	iron-oxygen complexes
LO_2^*	lipid peroxyl radical	HOCI	hypochlorite
LO^*	lipid alkoyl radical		
NO_2^*	nitrogen dioxide		
$^*NO$	nitric oxide		
RS^*	thiyl radical		
P^*	protein radical		

Free radicals and reactive oxygen species are produced by energy system or the antimicrobial defence system of the organism against external effects such as cigaratte smoke, unbalanced diet, food or environmental contaminants (Kumpulainen and Salonen, 1999). It is known that these active species and free radicals have a harmful effect due to causing various diseases including heart disease, cancer and aging (Noguchi *et al*., 1999).

Oxidative stress is the breakdown of the balance between oxido-reductive reactions, can be called as loss of oxidative homeostasis resulting in the excessive production of reactive oxygen species (Poljack-Blazi *et al*., 2010). The reasons of oxidative stress are due to decreased levels of antioxidants or increased production of reactive species (Halliwell, 2001).

When excessive amounts of reactive oxygen species exceed capacity of defense mechanisms against oxidative stress, this situation can cause serious cellular damage resulting in tissue injury or cellular death (Halliwell, 2001).

There are convincing arguments that oxidative stress cause many serious diseases. Atherosclerosis, arthritis, Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis are the examples of diseases that generally believed to be caused by oxidative stress (Di Giulio and Meyer, 2008).

Antioxidants are the major components of the defensive system from oxidation-caused damage. Shi *et al*. (2001) reported that there are several defence systems in vivo to decrease oxidative stress. The first of defence systems is preventive antioxidants responsible for diminishing the formation of free radicals. For instance, phospholipid hydroperoxide, peroxidase and catalase are examples that belong to that group. The second defence system includes radical-scavenging antioxidants which stop free radical chain reactions. Vitamin C, flavanoids, phenolic acids are the examples of this group. Another one is called as repair antioxidants removing the results of reactions between reactive species and biomolecules.

In foods, oxidative reactions result in deterioration of lipids. This process is called autooxidation and consists of three steps; initiation, propagation and termination. The autooxidation steps can be shown as below (Gordon, 2001).

Initiation:

$$RH \longrightarrow R^{*} + H^{*}$$

$$ROOH \longrightarrow RO^{*} + HO^{*}$$

$$2ROOH \longrightarrow RO^{*} + ROO^{*} + H_2O$$

Propagation:

$$R^{*} + O_2 \longrightarrow ROO^{*}$$

$$ROO^{*} \longrightarrow ROOH + R^{*}$$

Termination:

$$R^* + R^* \longrightarrow R\text{-}R$$

$$R^* + ROO^* \longrightarrow ROOR$$

$$ROO^* + ROO^* \rightarrow ROOR + O_2$$

In initiation step, lipid molecules transform into lipid radicals. After initiation step, one lipid radical forms another lipid radical, this step is called as propagation. In termination step free radicals react with other radicals (Gordon, 2001).

Antioxidants are subtances prone to donating hydrogen atoms. They do not only convert primary radicals to non- radicals but also prevent the reaction between lipid and radicals (Madhavi *et al*., 1996).

2.2 Phenolic Compounds

Phenolic compounds have one or more hydroxyl groups attached directly to an aromatic ring (benzene). They are typical compunds of plants and usually exist as esters or glycosides instead of free forms.

Compounds having more than one phenolic hydoxyl group attached to one or more benzene rings are called as "polyphenols" (Vermerris and Nicholson, 2008). These compounds are produced commonly by higher plants. They are responsible for essential organoleptic properties of plant-derived foods and beverages, especially colour and taste properties (Naczk and Shahidi, 2003).

Many of phenolic compounds have antioxidant activity in vitro and called as antioxidants. Categorization of phenolic compounds are given in Table 2.2 (Tokuşoğlu and Hall, 2011).

Table 2.2: Family of phenolic compounds (Tokuşoğlu and Hall, 2011).

Phenolic Compounds					
Phenolic acids	Flavonoids	Lignans	Stilbens	Tannins	Coumarins
Hydroxybenzoic acids	Flavons	Sesamol	Resveratrol	Hydrolyzed	
Hydroxycinnamic acids	Isoflavons	Sesamin	Piceatannol	Condensed	
	Flavonols	Sesamolin	Piceid		
	Flavanols	Sesamolinol	Pinoslyvin		
	Flavanones		Phapontisin		
	Anthocyanidins		Tamoxiphen		
	Anthocyanins		Derivative		
	Flavononols		Phytoalexins		
	Chalcons				

Polyphenols can represent antioxidant properties thanks to their redox potential. They can react as reducing agents, hydrogen donors, metal chelators and singlet oxygen scavengers. They have been used in processed foods as natural antioxidants instead of synthetic antioxidants so that they have gained much more attention (Rice-Evans *et al*., 1996).

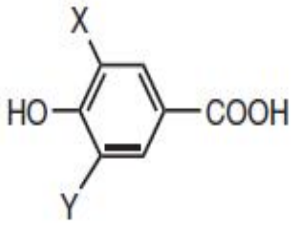
2.2.1 Phenolic acids

Phenolic acids are also known as hydoxybenzoates. Gallic, *p*-hydoxybenzoic, protocatechuic, and syringic acids are members of this group. They are generally not found as free forms, are usually found as components of complex structures like lignins or hydrolyzable tannins in the bond form (Jaganath and Crozier, 2010).

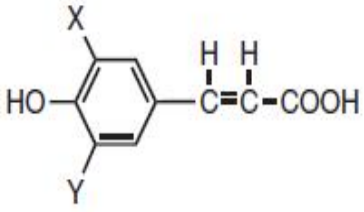
Phenolic acids are divided into two groups such as hydroxybenzoic acids and hydroxycinnamic acids. The major difference between these groups is the patterns of varied hydroxylations and methoxylations of their aromatic ring (Sarma, 2011).

The presence of a carbonyl group substitued on a phenol is a characteristic of hydoxybenzoic acids. Gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid, syringic acid and vanillic acid are some examples of that group. Caffeic acid, *p*-cumaric acid, sinapic acid and ferulic acid are examples of hydroxycinnamic acids. Hydroxycinnamic acids are generally found as esters of quinic acid, shikimic acid and tartaric acid such as chlorogenic acid that is an ester of caffeic acid and quinic acid (Vermerris and Nicholson, 2008). Among these phenolic acids, caffeic acids are

the major group representing over 75% of the total hydoxycinnamic acids in fruits (Baruah J. B., 2011). Figure 2.2 represents the classification of phenolic acids (Naczk and Shahidi, 2003).



a)Hydroxybenzoic acid



b) Hydoxycinnamic acid

Name	X	Y	Name	X	Y
p-hydroxybenzoic acid	H	H	Caffeic acid	OH	H
Gallic acid	OH	OH	*p*-cumaric acid	H	H
Protocatechuic acid	H	OH	Sinapic acid	CH_3O	CH_3O
Syringic acid	CH_3O	CH_3O	Ferulic acid	CH_3O	H
Vanillic acid	CH_3O	H			

Figure 2.1 : Classification of phenolic acids (Naczk and Shahidi, 2003).

Phenolic acids are believed to be response of the fruit to various kinds of stresses like mechanical, microbiological or chemical (Naidu *et al*., 2000).

2.2.2 Flavonoids

The basic structure of flavonoids includes two aromatic rings (A and B) linked through a three carbon bridge that is usually an oxygenated heterocycle (ring C), it can be seen in Figure 2.3 (Monfilliette-Cotelle, 2005). They can be found in free flavonoid aglycones in plants or as glycosided, methylated derivatives (Pietta, 2000).

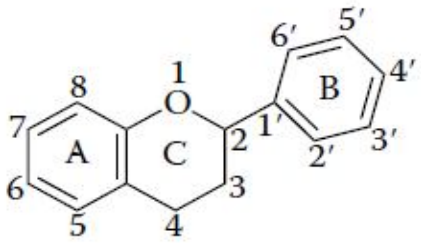


Figure 2.2 : Flavonoid backbone (Monfilliette-Cotelle, 2005).

Flavonoids can be categorized into six different groups depending on their structural difference. They include anthocyanins, flavonols, flavanols, flavanones, chalcones and isoflavones. The hydoxylation of the prone ring, presence of double bond, number and position of hydoxyl groups in the A and B ring are main factors of structural variance (Sarma, 2011). It is also possible to find different classifications of flavonoids in the literature. The Figure 2.4 represents different flavonoid groups.

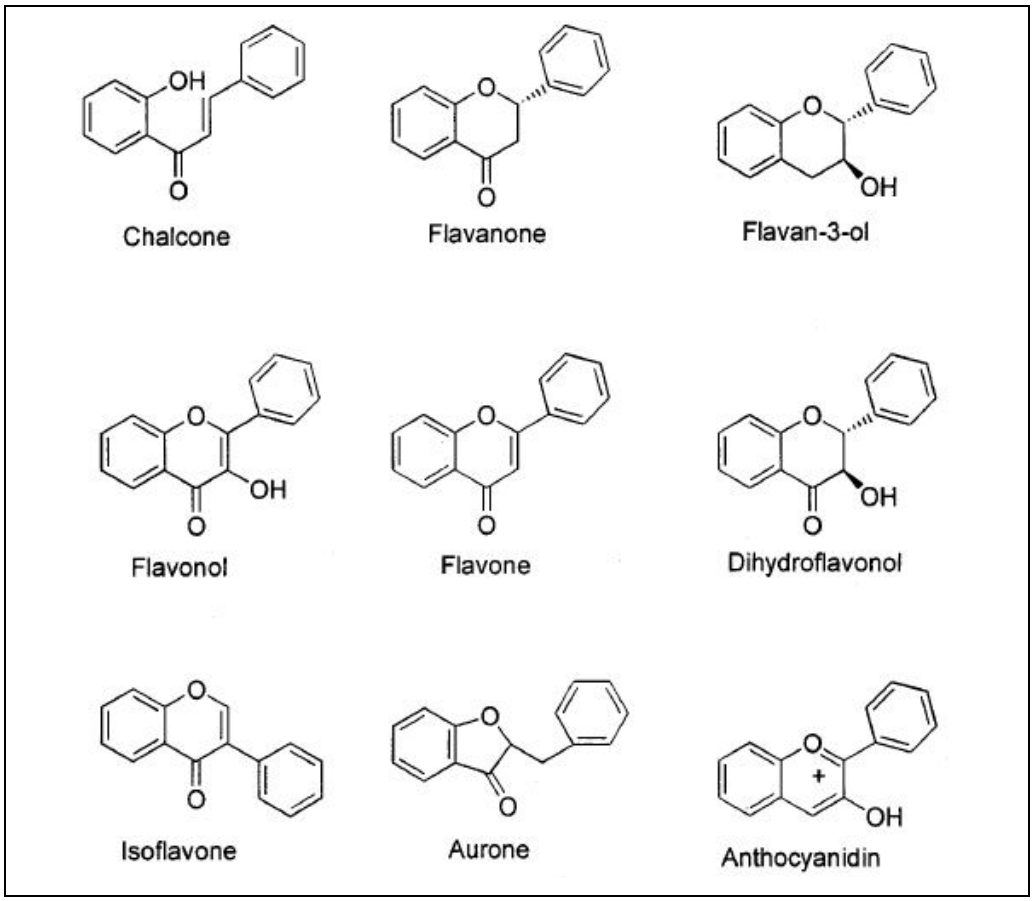
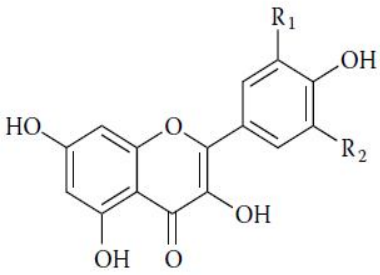
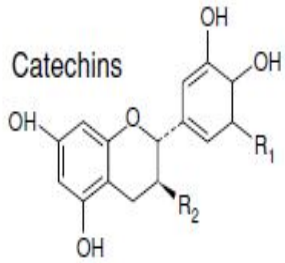


Figure 2.3: Flavonoids (Naidu *et al*., 2000).

Apples, grapefruits, berry fruits, orange, black/gren tea are considered as rich sources of flavonoids (Monfilliette-Cotelle, 2005).



a) Flavonols



b) Flavanols

Flavonol	R1	R2	Catechins	R1	R2
Quercetin	OH	H	Catechin	H	OH
Kaempferol	H	H	Epicatechin	H	H
Myricetin	OH	OH	Epigallocatechin	OH	OH

Figure 2.4 : Some examples of flavonols and flavanols (Kyle and Dythie, 2005).

It is known that flavonoid antioxidant actions have positive influence on human health and there are many mechanisms to explain the actions of flavonoid antioxidants (Disilvestro, 2000).

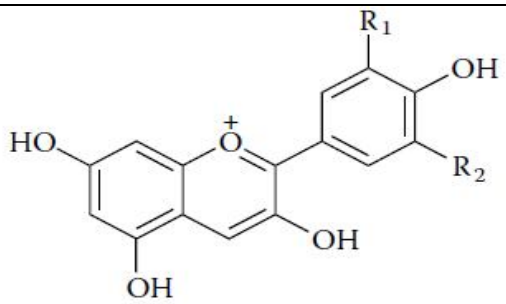
2.2.2.1 Anthocyanidins

Anthocyanidins are part of the large and widespread group of flavonoids and generally found as glycosylated or acylated form and responsible for red, pink or purple colours of plants. Water soluble glycosylated or acylated forms of anthocyanidins are called as anthocyanins. The number of anthocyanins are greater than the number of anthocyanidins because of the fact that each anthocyanidin can be glycosylated or acylated by varied sugars and acids (Mazza and Miniati, 1993).

Pelargonidin, cyanidin, delphinidin, malvidin, petunidin and peonidin are common athocyanidins in plants. The most common anthocyanidin found is cyanidin (Vermerris and Nicholson, 2008).

Glucose, galactose, rhamnose, rutinoside and arabinoside are common sugars bound to anthocyanidins. Researches have shown that anthocyanidins can be acylated with many hyroxybenzoic (*p*-hydroxybenzoic acid and gallic acid) and hydroxycinnamic acids (*p*-cumaric acid, ferulic acid and caffeic acid) as well (Mercadante and Bobbio,

2007). Figure 2.6 represents the chemical structures of different anthocyanidins (Monfilliette-Cotelle, 2005).



Anthocyanidin	R1	R2
Cyanidin	H	OH
Delphinidin	OH	OH
Malvidin	OMe	OMe
Pelargonidin	H	H
Petunidin	OMe	OH
Peonidin	OMe	H

Figure2.5: Chemical structure of anthocyanidins (Monfilliette-Cotelle, 2005).

Each six anthocyanidin has the basic structure of the flavylium cation (Vermerris and Nicholson, 2008). Nature and number of sugars attached to the flavylium cation are effective on stability and colours of anthocyanins (Mercadante and Bobbio, 2007).

The anthocyanins are able to impart colour to the plants and foods and this makes anthocyanins important compounds. In leaves, anthocyanins act as a light screen against UV radiation. Colour of anthocyanin depends on structure and concentration of pigment, temperature, pH, temperature, presence of copigments, metallic ions, enzymes, oxygen, ascorbic acid, sugars and their degradation products (Mazza and Miniati, 1993).

Anthocyanins can be found in the vacuoles of almost every cell type in tissues of all vegetative organs. They are to be found in roots, both subterranean and aerial and hypocotyls, stems, tubers, rhizomes and leaves (Hatier and Gould, 2009).

Anthocyanins can be found in different fruits and vegetables such as berries (blueberry, blackberry, chokeberry, raspberry, strawberry, etc.), onions, red cabbages, eggplants, grapes, currants in different amaunts (Clifford, 2000).

Since berries accumulate large quantity of anthocyanin between 0.1-10 mg /g sample, they are important anthocyanin sources in daily consumption. Dietary consumption of anthocyanins in U.S.A. was estimated at 215mg in summer and at 180mg in winter (Clifford, 2000).

2.3 Antioxidant Capacity Assays

It is known that antioxidants inhibit oxidative damage by inhibiting the reactive species, scavenging free radicals or increasing the internal antioxidant defences against oxidative stresss. Possessing all these advantages, it is important to determine the total antioxidant capacity of fruits and vegetables and the potential synergism among them (Salucci, 1999).

There are different methods used to determine the antioxidant capacity of herbal plants and foods. These methods can be divided into two groups; methods based on hydogen atom transfer (HAT) and methods based on electron transfer.

Oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP) are the examples of HAT based methods. ET based methods consist of total phenols assay by Folin-Ciocalteu reagent, ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] method, ferric ion reducing antioxidant power (FRAP), cupric ion reducing antioxidant assay (CUPRAC), free radical scavenging activity by DPPH (2,2-diphenyl-1picrylhydrazyl) (Albayrak *et al*., 2010). Principles of these assays can be found in Table 2.3 (Cao and Prior, 2001; Apak *et al*., 2011).

HAT based assays are based on measuring the quenching degree of free radicals by antioxidants. However, antioxidants react with fluorescent or oxidizing agents instead of free radicals in ET based antioxidant capacity assays (Apak R. *et.al*, 2011).

Table 2.3: Electron transfer based antioxidant capacity assays and their principles (Cao and Prior, 2001; Apak *et al*., 2011).

Assay	Principle	Wavelength
ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] assay	Measurement of inhibition of the absorbance of ABTS•+ radical cation by reductants	660,734,820nm
CUPRAC (Cupric ion reducing antioxidant capacity) method	Measurement of orange-yellow color of reduced [Cu+-Neocuproine	450nm
DPPH(2,2-diphenyl-1picrylhydrazyl) assay	Evaluation of scavenging activity of antioxidants by measurement of change in absorbance	515,517nm
FRAP (Ferric reducing ability of plasma) assay	Measurement of blue color of reduced [Fe2+-TPTZ tripyridyltriazine] at low pH	593nm
Total Phenols Assay (Folin Method)	Measurement of reduction of Mo(VI) to Mo(V)	765nm

2.4 Blueberry *(Vaccinium arctostaphylos L.)*

Blueberries, *Vaccinium* is a member of *Ericaceae* family. They generally grow in wild parts of the world. However, it is used by the food industry especially in Canada and United States. Blueberries grow best in acidic soil. There are many types of blueberries including higbush blueberry, lowbush blueberry, rabbiteye blueberry and caucasian blueberry (Mazza and Miniati, 1993).

Blueberries were reported to have anticancer effects due to their ability to reduce oxidative stress and remedy the negative effects of oxidative stress. Blueberries were known to be serious phytochemical sources and these phytochemicals also block treatment resisting pathways of tumor cells (Seeram N., 2006).

Blueberries contain high amounts of phenolic compounds including anthocyanins, flavonols, proanthocyanidins and phenolic acids (Moyer et. al., 2002; Sellapan *et al*., 2002; Cho *et al*., 2005; Koca and Karadeniz, 2009; Lättı *et al*.,2009).

Different types of cultivated blueberries are rich sources of varied phenolic components as shown in Table 2.4. Cho *et al* (2005) reported quercetin 3-galactoside to be the predominant flavonol in Apache, Arapaho, Kiowa and Navaho types. However, quercetin 3-glucoside was the most important one in Prime-Jan Chicksaw types. Table 2.4 represents the anthocyanins found in blueberries (Mazza and Miniati, 1993).

Table 2.4 : Anthocyanins found in blueberries (Mazza and Miniati, 1993).

Anthocyanins				
Cyanidin3-glucoside	Delphidin 3-glucoside	Malvidin 3-glucoside	Peonidin 3-glucoside	Petunidin 3-glucoside
Cyanidin 3-galactoside	Delphidin 3-galactoside	Malvidin 3-galactoside	Peonidin 3-galactoside	Petunidin 3-galactoside
Cyanidin 3-arabinoside	Delphidin 3-arabinoside	Malvidin 3-arabinoside	Peonidin 3-arabinoside	Petunidin 3-arabinoside

The synthesis of anthocyanins and other phenolic compounds are influenced by several environmental factors (Kalt *et al*., 2001). Growing region, season and cultivation techniques can be thought as environmental conditions affecting antioxidant capacity, phenolic and anthocyanin content of blueberries. Many biotic and abiotic factors change year by year and affect the amount of phenolic compounds in berries. Furthermore increased light intensities result in higher phenolic contents (Wang, 2007).

It was represented that storage conditions have severe influence on phenolic contents of blueberries because of the fact that phenolic compounds are unstable and is likely to be lost at high temperatures (Srivastava, 2007). Srivastava (2007) studied the effect of storage temperature on blueberry exract packed in glass bottles (-20ºC, 6ºC, 23ºC and 35ºC) and found that storage conditions have serious influence on phenolic compounds. The study illustrated that there was no significant loss of total phenolic, anthocyanin content and antioxidant capacity of samples at -20ºC at the end of 30 days period. However, storage at higher temperatures resulted in loss of bioactive components. Storage in frozen state or storage at low temperatures was suggested to prevent the phytochemical compounds of blueberry.

A study carried out by Sellapan *et al*. (2002) investigated blueberries and blackberries grown at different locations in Georgia. Samples were analyzed for total flavonoid, anthocyanin, polyphenols and trolox-equivalent antioxidant capacity. Moreover, phenolic profile of samples was analyzed for gallic acid, *p*-hydroxybenzoic acid, caffeic acid, *p*-cumaric acid, ferulic acid, ellagic acid, catechin, epicatechin, myricetin, quercetin and kaempferol. Phenolic acid content was found to

be in the range of 0.19- 258.90 mg phenolic acid/100g fresh weight. Total phenolic content was expressed as gallic acid equivalents and results showed that samples have a phenolic content ranging from 2.61 to 9.29 mg GAE/g fresh sample. The anthocyanin content was found to be 0.12-1.97 mg cyanidin 3-glucoside equivalents (C3GE) changing up to cultivar and sample location. Catechin was found to be the major flavonoid found in blueberries with concentrations up to 3.87mg/g fresh sample.

Ehlenfeldt and Prior (2001) reported phenolic and anthocyanin concentrations in fruit tissues of 87 highbush blueberries.The average values were found to be 1.79 mg GAE/g and 0.95 mg C3GE/g sample for total phenolic and anthocyanin content respectively. The study illustrated that blueberry fruits and leaf tissues had a wide range of phenolic content.

Cho *et al*. (2005) studied HPLC profile of flavonol glycosides of different blueberry genotypes. The results of total flavonol content was expressed as rutin equivalent and showed that total flavonol contents of samples were between 190 mg/kg-320mg/kg. The total phenolic content of genotypes ranged from 2.269-3.699 mg GAE/g sample. Quercetin derivatives were found to be the dominant flavonols in blueberry, possesing 75% of total flavonols.

According to literature data, polyphenolics are located generally on the skin of berries so that small- fruited berries have more anthocyanins than big ones due to their higher skin areas (Gao and Mazza, 1994).

Moyer et. al. (2002) investigated 107 different fruits of *Vaccinium* species to determine the anthocyanin, phenolic content and antioxidant capacity using ORAC method. Extraction of samples were carried out using acetone:water (70:30, v/v). Anthocyanin contents ranged from 34 to 515 mg C3GE/ 100g; ORAC values of samples ranged from 19-131µmol TE/g. High correlation was observed between berry size and anthocyanin content for higbush blueberry samples. It was assumed to be related with pigment location on the skin in highbush types.

Blueberries are popular berries in the Black Sea Region of Turkey. Wild types were used to be consumed in past. At present , cultivated berries are grown in larger areas as a result of breeding studies. Wild and cultivated blueberries were analyzed for total anthocyanin, phenolic content and antioxidant activity using FRAP method by

Koca and Karadeniz (2009). A mixture of different solvents acetone/methanol/water/formic acid (40:40:20:0.1, v/v/v/v), was used to exract phenolic compounds. Total anthocyanin content was ranged from 0.18- 2.94 mg C3GE/g fresh sample. Total phenolic content was ranged from 0.77- 5.42 mg GAE/g fresh sample. In this study, wild blueberry types were found to possess higher antioxidant values than cultivated types (Koca and Karadeniz, 2009). The *Vaccinium arctostaphylos L.* can be seen in Figure 2.7 (Çelik, 2009).

Figure 2.6: *Vaccinium arctostaphylos L.* (Çelik, 2009).

Vaccinium arctostaphylos L is a blueberry type grown in the Black Sea Region of Turkey and called as "Caucasian Blueberry" (Çelik et. al, 2008). Lättı *et al*. (2009) studied the characteristic anthocyanins in Caucasian Blueberries selected from northeastern Anatolia. The average anthocyanin content of samples was found to be 14.20 mg C3GE/g dry sample.

Delphidin was found to be the predominant anthocyanidin (41%) followed by petunidin (19%) and malvidin (19%). Characteristic anthocyanins of Caucasian blueberries can be seen in Table 2.5 (Lättı *et al*., 2009).

Table 2.5: Characteristic anthocyanins of Caucasian blueberries (Lättı *et al*., 2009)

Anthocyanins	
Delphinidin galactoside	Dephinidin pentoside
Delphinin hexose-pentoside	Petunidin arabinoside
Delphinidin glucoside	Peonidin glucoside
Cyanidin galactoside	Malvidin galactoside
Delphinidin arabinoside	Peonidin arabinoside
Cyanidin glucoside	Malvidin glucoside
Petunidin galactoside	Malvidin arabinoside
Cyanidin arabinoside	Petunidin pentoside
Petunidin glucoside	Peonidin pentoside
Peonidin galactoside	

2.5 Oat

Oats (*Avenia sativa L.)* have been consumed by humans since ancient times and accepted as a healthy food without a fully understanding of its health promoting effects. In recent years, oat has gained public attention and consumption of oat and oat-derived products has showed a growing trend due to the findings on its hypocholesterolemic and hypoglycemic effects (Mälkki, 2001).

Oats contain a wide range of phenolic components including free or bounded forms (Dimberg and Jastrebova, 2009). Ferulic acid is an example of phenolic acid fractions existing as a major phenolic acid in oats. In addition, the presence of many other phenolic acids such as free *p*-coumaric and vanillic acids and vanillic, sinapic, *p*-coumaric, *p*-hydroxyphenylacetic, caffeic, protocatechuic, syringic and *p*-hydroxybenzoic acids in bounded forms were reported (Naczk and Shahidi, 2003). Kovačova and Malinova (2007) investigated 21 oat genotypes and ferulic and coumaric acids were found to be the predominant phenolic acids found in the samples.

An oat kernel consist of hulls (20-35%) which are the unproceessed parts of kernel. Hulls include approximately 85% of insoluble fiber. The groat, which is the "part without hull", contains 6-9 % of dietary fiber. Dietary fiber can be found in the tissues outside the aleurone layer. The major soluble fiber compound is a linear polysaccharide ß-glucan located in endosperm cell walls. It is reported that genetic

and environmental factors have influence on ß-glucan content of oat groats, changing from 1.8 to 8.5 %. Hulled and heat treated groats are used to prepare oat flakes. The process consists of a rolling step applied by rolling between castrion rolls, and a further steaming step aiming to inactive enzymes and plasticise the groats (Mälkki, 2001).

Some researchers reported the total phenolic contents of different oat-based breakfast cereals (Ryan *et al*., 2011; Yu *et al*., 2002). Yu *et al* (2002) found the total phenolic content of four different oat-based breakfast cereal ranging from 0.203- 0.504 mg GAE/g sample. Ryan *et al*. (2011) studied 30 different commercial oat-based breakfast cereal containing various fruits such as apple, blueberry and total phenolic content was found to be between 1.506-1.853 mg GAE/g sample.

2.6 Milk

Milk includes various kinds of proteins possesing biactive properties. These bioactive properties include antimicrobial, antihypertensive, antioxidative, anticytotoxic activites (Park Y., 2009).

The protein fraction can be divided into two groups such as caseins and whey proteins. Although the casein proteins contribute to the major protein content of milk,both casein and whey proteins have influence on antioxidant capacity (Rival *et al*., 2001; Chen *et al*., 2003).

Chen *et al*. (2003) studied total antioxidant capacity of bovine milk using spectrophotometric methods. The bovine milk, whey and low molecular weight fraction of whey were investigated for their total antioxidant capacity using ABTS and FRAP assays. Casein was reported to be the most important ABTS scavenger in milk due to the presence of high contents of potentially antioxidative amino acids like tyrosine, tryptophan, histidine, lysine and methionine. FRAP method was reported to be an unsuitable method for high molecular weight (HMW) fractions from whey. It was reported to be only suitable for determining the antioxidant activity of low molecular weight (LMW) fractions. As usually recommended different antioxidant capacity methods was suggested to get a better understanding.

Rival *et al*. (2001) stated that casein and their hydrolysates were able to inhibit enzymatic and non-enzymatic lipid peroxidation. It was stated that protein and fatty

acid fraction could possess antioxidant capacity by acting as a target to oxidative degredations.

2.7 Matrix Effect

Many foods such as coffee, tea, breakfast cereals, infant cereals are consumed with milk. Researches were conducted in order to asses the effect of addition fo milk, skimmed-milk or semi-skimmed milk on antioxidant capacity of tea when consumed together with milk.

Hasni *et al*. (2011) reported that there was a weak bonding between tea polyphenols and α-casein, ß-casein in solutions. In this study,catechin(C), epicatechin (EC), epigallocatechin (EGC) and epigallocatechin gallate (EGCG) was investigated for their binding properties to α-casein, ß-casein and it was illustrated that higher the OH containing groups the more prone to bonding (E ~C> EC >EGC> EGCG). In addition, it was revealed that ß-casein fractions create stronger bonds than α-casein fractions due to their hydrophobic character.

Dupas *et al*. (2006a) have studied the effect of milk on coffee samples and reported that there was no significant effect of milk addition on antioxidant activity of coffee samples. Moreover it was concluded that fat content of milk had also no influence on antioxidant activity.

Sharma *et al*. (2007) studied the effect of milk and sugar addition to tea and as a result black tea showed highest antioxidant activity compared to tea and milk, tea+milk+sugar and tea+sugar mixtures. Another study conducted by Langley-Evans (2000) showed that there was a significant effect of milk on antioxidant capacity of tea samples. Greatest inhibition was observed in black tea+whole milk mixtures (28% reduction) compared to black tea+semi-skimmed milk (22% reduction) and black tea+skimmed milk (12% reduction).

In an in vitro study, full fat, semi-skimmed and skimmed milks were added to blueberry extracts. Decrease in antioxidant capacity values were observed for blueberry extracts and full-fat milk was found to be responsible for the highest inhibition (Serafini *et al*., 2009).

2.8 Bioavailability

The bioavailability term has various definitions. The bioavailability term originates from pharmacology and it is explained as "rate and extent to which a drug reaches its site of action" It refers to the amount of ingested quantitity of a nutrient or a compound that reach the systematic circulation. Not only the identification and quantification of existing polyphenols in food samples is crucial but also investigation of their bioavailability and biological activity on tissues or cells is also important in order to determine the effetcs of polyphenols on prevention of diseases (D'Archivio *et al*., 2010).

The bioavailability term consists of several sequential steps. It includes the availability for absorption, metabolism, tissue distribution, and bioactivity. The bioactivity measures the biologic activity of components on specific organs or tissues. On the other hand, it is not easy to determine the bioactivity so that bioavailability term is usually used in a narrow sense. It is referred as the part that enters the blood stream from a consumed dose of a nutrient or metabolite. The bioavailability and bioaccessibility terms can often be confused but bioaccessibility term only refers to the proportion that is being available to be absorbed from intestinal tract. However, bioavailability term includes transportion of components to cells and bioactivity term as well (Fernández-García *et al*., 2009). Table 2.6 represents the steps of bioavailability (Fernández-García *et al*., 2009).

Table 2.6: Steps of bioavailability (Fernández-García *et al*., 2009).

BIOAVAILABILITY	
BIOACCESSIBILTY	BIOACTIVITY
• Events that take place during food digestion • Absorption/Assimilation through epithelial tissue • Pre-systemic metabolism	• Transport and assimilation by the target tissue • Interactions with biomolecules • Metabolism or biotransformation • Physicological Response

Bioaccessibility studies can be divided into two parts as experiments conducted as in vivo and vitro models. In vivo assays which include animal models was stated that

they could not model and completely simulate the absorption and metabolism of caretenoids in humans even if animal models are more reasonable than human studies (Fernández-García *et al*., 2009).

Two assays were mentioned to assess the bioaccessibility of nutrients in vitro. First one is in vitro digestion process consisting of oral, gastric and small intestinal digestion. Second one involves the use of Coco-2 cell lines examining the accessibility for absoption and cellular metabolism. It was reported that two methods both have advantages and disadvantages (Failla *et al*., 2008).

In vitro digestion method is more cheaper and easy to handle according to Coco-2 cell method. The method needs no extra equipment apart from standart laboratory equipment. The method can be controlled easily and it is possible to investigate the mechanisms. On the other hand, the method's sensitivity is low due to composition and quantity of test samples. Regarding Coco-2 cell method, showing similar phenotype to normal absorptive epithelial cells and growing on dish surface and on membrane are assets of this method. Moreover, it is possible to get a response, cells synthesize and secrete chylomicrons in response to prandial state. Since cells are obtained from human colonic adencarcinoma, presence of other epithelial cell types and humoral factors in small intestine may affect enterocyte activities (Failla *et al*., 2008).

Several factors have influence on bioavailability of these assays. These variables can be related with environmental conditions, food properties and food processing conditions or can be related to host or polyphenols and also it is likely to occur interaction between compounds (D'Archivio *et al*., 2010). The variables affecting bioavailability can be seen in Table 2.7.

In vitro digestion method was used to determine the potential bioavailability of phenolic components of various food samples by several researchers (Gil-Izquierdo *et al*., 2001; McDoughall *et al*., 2005, 2007; Failla *et al*., 2008; Liang *et al*., 2011).

Table 2.7 : Variables affecting bioavailability of phenolics (D'Archivio *et al*., 2010).

Environmental Factors	• Sun exposure • Different types of culture • Fruit yield for tree • Rainfall
Factors related to food process	• Heat treatment • Cooking technique • Storage conditions • Homogenization
Factors related to food	• Food matrix (interaction between polyphenols and proteins, carbohydrates, etc.)
Interaction with other compounds	• Binding to blood proteins (For instance, serum albumin)
Factors related to polyphenols	• Free or bound forms (glycosilated forms) • The quantity
Factors related to host	• Systemic factors (gender and age; disorders and genetics) • Intestinal factors (colone micoflora, enzyme activity)

Gil-Izquierdo *et al*. (2001) studied the in vitro flavonoid bioaccessibility of orange juice by using an in vitro method stimulating gastric and small intestinal digestion. The results showed that a small part of orange juice flavanones are available for absorption. However, it was stated that it does not mean that insoluble part was lost. It was reported that insoluble part was being transmitted to large intestine and could be transformed by colon microflora.

McDoughall *et al*. (2005) investigated potential bioavailability of anthocyanins from raspberry exracts and the effect of codigestion on bioavailability. After gastric digestion, samples were referred as "POSTGASTRIC (PG)". After small intestinal digestion , the sample outside the dialysis tube was referred as the "OUT" sample and the sample leftin the dialysis tube was referred as the "IN" sample.Results illustrated that anthocyanins could be recovered from PG sample. However, only a

small percentage of anthocyanins (5%) could enter IN sample. The sum of OUT and IN samples was about 70% according to initial sample. It was argued that codigestion had a negative influence on PG and OUT samples. On contrary, it had a positive influence on IN samples.

The mulberry (*Morus atropurpurea Roxb*.) samples were investigated for their antioxidant capacity and bioaccessibility through gastrointestinal in vitro digestion procedure. The digest showed a higher antioxidant acitivity because of the phenolics resulted from degradation of anthocyanins under intestinal environment. However, the bioaccessibility of anthocyanins were found to be decreased excessively. Recovery from the IN sample was 0.34% whereas recovery from the OUT sample was only 4.58 % (Liang L *et al*., 2011).

The red wine was also investigated for its anthocyanin stability through in vitro digestion procedure. The anthocyanins were found to be stable in gastric conditions but important losses were detected during intestinal digestion. Before gastrointestinal digestion, red wine was reported to have over 20 identifiable anthocyanins but only five of them survived at the end of gastrointestinal digestion and detected in IN and OUT samples (McDoughall *et al*., 2005).

Gião *et al*. (2012) used an in vitro digestion/Coco-2 cell culture model to evaluate the bioavailability of phenolic components of aqueous extracts of *Agrimonia eupatoria, Rubus idaeus, Salvia sp.* and *Satureja montana.* In vitro digestion procedure also included mouth digestion in addition to gastric and intestinal digestion. It was reported that some phenolics were not affected from digestion procedure but some of them faced with important losses. Rutin was found to pass the Coco-2 cell barrier. The most stable compound throughout the in vitro digestion was 3,4,5-trihydroxybenzaldehyde which was followed by chlorogenic acid and rutin, respectively

3. MATERIALS AND METHODS

3.1 Blueberry, oat meal and milk samples

Blueberry *(Vaccinium arctostaphylos L.)* samples were collected from Eynesil, Giresun (41° 3' 37" North, 39° 8' 33") of Turkey. They are also called as "Caucasian whortleberry". *Vaccinium* samples were packed immediately and stored in a freezer at -20º C until analyzed. Oat meal, skimmed milk (0.1% fat; 3.3% protein), whole milk (3.4% fat; 3.1% protein content), were purchased from a local supermarket in İstanbul. (*Vaccinium arctostaphylos L.)* can be seen in Figure 3.1.

Figure 3. 1 : Blueberry (*Vaccinium arctostaphylos L.)*

3.2 Preparation of Food Matrices

One variety of *Vaccinium arctostaphylos L.*was used for the preparation of food matrices. Blueberry, whole milk or skimmed milk and oat meal were blended together at specific ratios [(4:1:8) for oat meal/blueberry/milk; w/w/w] in order to prapare a breakfeast cereal that's similar to consumers' habits for consumption.

Percentage of the ingredients (oat meal/blueberry/milk) in the respective blends on weight basis are given in Table 3.1. The samples were also coded.

Table 3.1 : Percentages (%) of the ingredients (based on weight basis).

Code	Ingredients	Oat Meal (%)	Blueberry (%)	Whole milk (%)	Skimmed milk (%)	Total (%)
O	Oat meal	100				100
B	Blueberry		100			100
M1	Whole milk			100		100
M2	Skimmed milk				100	100
OM1	Oat meal+milk	33.3		66.6		100
OM2	Oat meal+skimmmed milk	33.3			66.6	100
BM1	Blueberry+milk		11.1	88.9		100
BM2	Blueberry+skimmed milk		11.1		88.9	100
OB	Oat Meal+blueberry	80.0	20.0			100
OBM1	Oat Meal+blueberry+milk	30.8	7.7	61.5		100
OBM2	OatMeal+blueberry+ skimmed milk	30.8	7.7		61.5	100

Preparation of blends was repeated three times and all the analysis were performed in triplicates. Ingredients and mixtures were further chilled in liquid nitrogen and ground to powder using a analytical mill (IKA A11 basic, Germany). Chilled test tubes were filled with powdered samples and then stored at in a freezer at -80º C until analyzed. Nitrogen milling were used to decrease enzyme activity and oxidation in the study.

3.3 Chemicals

Acetone (≥99.8%), ethanol (≥99.8%), Folin Ciocalteu reagent, catechin (≥98%) , neocuproine (Nc), DPPH (2,2- diphenyl- picrylhydrazyl), TPTZ (2,4,6-tripyridyl-2-triazine), pepsin, pancreatin, bile salts, dialysis tubes, acetonitrile (99.8%), quercetin-3-ß-D-glucoside (≥98%), cyanidin-3-o-rutinoside (≥98%), pelargonidin-3-O-glucoside (callistephin chloride) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Acetic acid, methanol (≥99.9%) formic acid (≥98%), sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), sodium hydroxide (NaOH), hydrochloric acid (HCI) (37%), sodium acetate ($CH_3CO_2Na{\cdot}3H_2O$), potassium persulfate ($K_2S_2O_8$), copper (II) chloride ($CuCl_2{\cdot}2H_2O$), ammonium acetate (CH_3COONH_4) and trifloroacetic acid (TFA, 99%), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4) were purchased from Merck KGaA (Darmstadt, Germany). Gallic acid (≥99%), cyanidin-3-O-glucoside (kuromanin chloride), ≥96%), cyanidin chloride (cyanidin 3,5-di-O-glucoside), delphinidine chloride (delphinidin 3-O-glucoside), pelargonin chloride (pelargonidin 3,5-di-O glucoside), delphinidin 3-glucoside, malvidin-3-galactoside, peonidin-3-

glucoside and petunidin chloride were purchased from Extrasynthese (Genay, France). Aluminum chloride ($AlCI_3$), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), chlorogenic acid (≥98%), p-coumaric acid (≥98%), ferulic acid (≥98%), quercetin-3-galactoside (≥98%) and caffeic acid (≥95%) were purchased from Fluka Chemie (Buchs, Switzerland). Potassium chloride (KCI) was purchased from Riedel-de Haen Laborchemikalien GmbH (Hanover, Germany) and ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)) was purchased from Applichem GmbH (Darmstadt, Germany). Ferric chloride ($FeCl_3$) was purchased from Lachema (Czech Republic) and sodium bicarbonate ($NaHCO_3$) was purchased from BDH Chemicals Ltd (Poole, UK).

3.4 Methods

Methods used to determinate total phenolic, total flavonoid, total anthocyanin content and to investigate antioxidant capacity and potential bioavailability of the individual ingredients together with the blends were mentioned below.

3.4.1 Exraction method for the samples

The exraction procedure was carried out with acetone:water (70:30, v/v) as described by Ryan et.al. (2011) with some few modifications. Extractions were carried out triplicate for each sample. 2 g of each sample was extracted with 20 mL of acetone:water (70:30, v/v) solvent at three steps. First, 2 g of sample was extracted in 10 mL solvent for 15 min at 4 ºC using a ultrasonic bath (VWR Ultrasonic Cleaner, Malaysia) and then, the extracts were centrifuged at 4000 rpm for 10 min at 4 ºC using a bench type centrifuge (Hettich Zentrifugen D78532, Germany). The supernatant was collected. This step was repeated three times.The extracts were then stored at -20 ºC until the analyses. The supernatant was used for determining antioxidant activity, total phenolic, flavonoid and anthocyanin contents of the samples.

For HPLC profile determination, methanol:water:formic acid (75:25:0.1) extracts of the samples were prepared following the same extraction steps as mentioned above.

3.4.2 Total phenolic content

The total phenolic content was determined according to Folin Ciocalteu method as described by Ryan *et al*. (2011) with some few modifications. Folin Ciocalteu reagent was diluted ten times with milli-Q water The sample extract (100µL) was mixed with 0.75 mL of Folin Ciocalteu reagent (1:10 v/v, with water). The mixture was allowed to equilibrate for 5 min and then mixed with 0.75 mL of 60 g/L sodium carbonate solution. After incubation at room temperature for 90 min, the absorbance was measured at 725 nm using 70% acetone as the blank. Calibration curve was prepared with gallic acid stock solutions using 0.01-0.4 mg/mL gallic acid concentratrations. The calibration graph can be seen in Appendix A. The results were expressed as mg of GAE per gram of fresh sample. All analyses were repeated three times for each extract.

3.4.3 Total flavonoid content

Total flavonoid content analysis was performed colorimetrically according to Kim et. al. (2003). Firstly, 1mL of sample extract was mixed with 0.3 mL of 5% $NaNO_2$ solution, waited for following 5 minutes and then 0.3 mL 10% $AlCI_3$ was added. After one minute, 2mL of 1 M NaOH was added and the total volume was adjusted to 6 mL by adding distilled water. Samples were vortexed for 10 seconds and absorbance was measured immediately at 510 nm against a reagent blank using UV-Visible Spectophotometer (Shimadzu UV-1700 Pharmospec, Japan). Calibration curve was prepared by using catechin standard at 0.01-0.1 mg/mL catechin concentrations and calibration graph was given in Appendix A. The results were expressed as catechin equivalent per gram of fresh sample.

3.4.4 Total anthocyanin content

Total anthocyanin content was determined with AOAC Offical Method 2005.02 using pH differential method. Firstly, 1.86 g of KCI was weighed in to a beaker and dissolved in water. The pH was adjusted to 1.0 with concentrated HCI (37%) and the final volume was adjusted to 1 L with distilled water. Secondly, 54.43 g of $CH_3CO_2Na.3H_2O$ (sodium acetate) was weighed and dissolved in water. The pH was adjusted to 4.5 with concentrated HCI (37%) and the final volume was adjusted to 1L with distilled water. The samples were diluted with buffers pH 1.0 and pH 4.5) at reasonable ratios. The absorbances of the dilutions were measured at 520 nm and 700

nm against distilled water using UV-Visible Spectophotometer (Optima SP-3000 nano, Japan). Results were expressed as cyanidin-3-glucoside basis as calculated according to the equation given below.: The results were then converted to mg cyanidin-3-glucoside /g fresh sample.

Anthocyanin content (cyanidin-3-glucoside equivalents, mg/L) =

$$(\Delta A * MW * DF * 1000)/(\varepsilon * L) \quad \textbf{(3.1)}$$

$$\Delta A = (A \text{ at } 520 \text{ nm} - 700 \text{ nm})_{KCl} - (A \text{ at } 520 \text{ nm} - 700 \text{ nm})_{CH3CO2Na \cdot 3H2O} \quad \textbf{(3.1a)}$$

Where,

A: Absorbance

MW: Molecular weight for cyanidin-3-glucoside (449.2 g/mol)

DF: Dilution factor

ε: Molar extinction coefficient for cyanidin-3-glucoside (26900)

L: Path length, cm

3.4.5 Antioxidant capacity assays

Antioxidant capacity of samples were determined by FRAP (Ferric reducing ability of plasma), DPPH (2,2- diphenyl-1-picrylhydrazyl), CUPRAC (Cupric ion reducing antioxidant capacity) and ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] assays. All experiments were performed in triplicates. Trolox prepared in acetone was used to prepare calibration curves for antioxidant capacity methods. The results were expressed as µmol trolox equivalent/g of fresh sample.

3.4.5.1 ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] assay

Analysis was carried out as described earlier by Miller and Rice Evans (1997) with some modifications. Before the analysis, ABTS solution, potassium persulfate ($K_2S_2O_8$) solution and 0.05 M potassium phosphate buffer were prepared. Potassium phosphate buffer (pH = 8) (0.05 M KPi buffer) was prepared by mixing 0.05 M potassium dihydrogen phosphate (KH_2PO_4) with 0.05 M dipotassium hydrogen phosphate (K_2HPO_4). ABTS solution was prepared by dissolving 220 mg of ABTS in 200 mL of milli-Q water. Potassium persulfate was prepared by dissolving 38 mg of $K_2S_2O_8$ in 2mL of milli-Q water. ABTS stock solution was prepared by mixing

potassium persulfate and ABTS solution and storing the final solution overnight in dark. ABTS stock solution was diluted with 0.05 M potassium phosphate buffer until absorbance had reached 0.9±0.2. Then, 100 mL sample was mixed with ABTS working solution. After one minute, the absorbance was measured at 734 nm using UV-Visible Spectophotometer (Shimadzu UV-1700 Pharmospec, Japan). The results were expressed as milimolar trolox equivalent values. Calibration curve was prepared by using trolox reagent in the presence of acetone at 0.005-0.10 mg/mL concentrations and calibration graph was given in Appendix A. All analyses were repeated three times for each sample extract.

3.4.5.2 CUPRAC (Cupric ion reducing antioxidant capacity) assay

CUPRAC (Cupric ion reducing antioxidant capacity) assay was performed as described by Apak et. al. (2004). Firstly, 10 mM copper (II) chloride solution, ammonium acetate buffer (pH 7.0) and neocuproine solution in 96% ethanol were prepared. After than,, 1 mL of each solution were added to a test tube followed by the addition of 1 mL of distilled water and 100 µL of sample extract. The mixture was vortexed andafter incubating at room temperature for an hour, absorbance measurements were performed against a reagent blank at 450 nm. Calibration curve was prepared by using trolox reagent in the presence of acetone at 0.01-0.8 mg/mL concentrations and given in Appendix A. The results were expressed as µmol trolox equivalent/g fresh sample.

3.4.5.3 DPPH (2,2- diphenyl-1-picrylhydrazyl) assay

The procedure was carried out as described by Kumaran and Joel karunakaran (2006) with some modifications. Prior to analyses, 0.1 mM of DPPH solution was prepared by dissolving 0.0039 mg of DPPH (2,2- diphenyl- picrylhydrazyl) in methanol. To carry out procedure, 2mL of DPPH (2,2- diphenyl-1-picrylhydrazyl) was added to 100µL of sample extract and vortexed for 20 sec. Absorbance measurements were performed after incubation in dark for 30 min at 517nm using UV-Visible Spectophotometer (Shimadzu UV-1700 Pharmospec, Japan). Trolox at different concentrations in the range of 0.01-0.4 mg/mL prepared in acetone was used to obtain calibration curve and the calibration curve was given in Appendix A. The results were expressed as µmol trolox equivalent/g fresh sample. All analyses were performed triplicate for each extract.

3.4.5.4 FRAP (Ferric reducing ability of plasma) assay

FRAP (Ferric reducing ability of plasma) assay was performed according to Deighton et. al (2000) with some modifications. 0.3 M sodium acetate solution, 10 mM TPTZ (2,4,6-tripyridyl-2-triazine) solution and 20 mM ferric chloride solution were prepared with a ratio of 10:1:1,v/v/v respectively to prepare FRAP reagent. Procedure was performed by mixing 100 µL of sample exract with 900 µL of FRAP reagent then samples were vortexed for 20 sec. Absorbance measurements were carried out at 593 nm exactly after 4 min following mixing the sample and FRAP reagent. Trolox at different concentrations in the range of 0.001-0.1 mg/mL prepared in acetone was used to obtain calibration curve. The calibration graph was given in Appendix A. The results were expressed as µmol trolox equivalent/g fresh sample.

3.4.6 In vitro digestion assay

The procedure was performed as described by McDoughall et. al (2005). Two consecutive steps were applied as described below. Figure 3.2 represents bioaccessibility assay to determine the potential bioavailability.

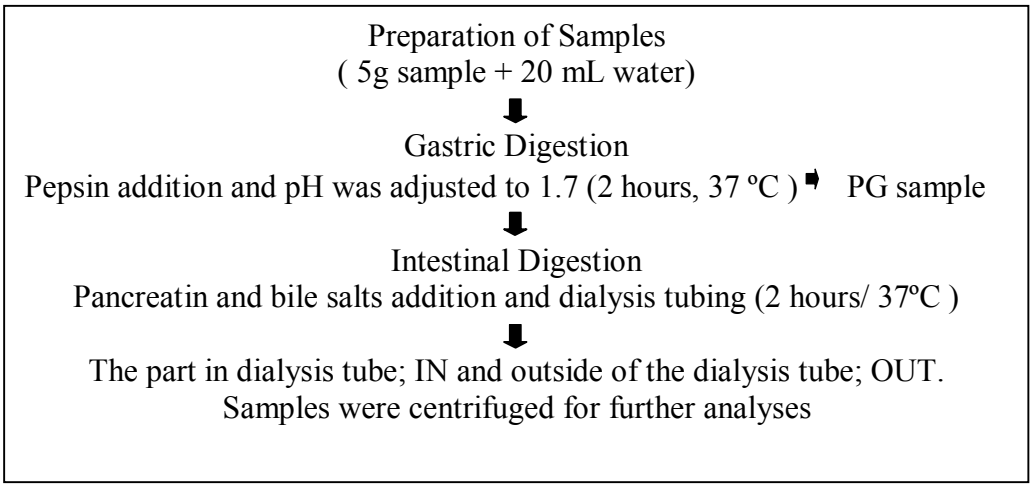


Figure 3. 2 : Bioaccessibility procedure (McDoughall *et al*., 2005).

In the first step, digestion in the stomach was simulated. For this step, 5 g of grounded sample was weighed and 20 mL distilled water and 1.5 mL of pepsin solution with a concentration of 1mg/mL pepsin were added to samples and 5 M HCI was used to adjust pH to 1.7. Then, sample was allowed to incubate at 37ºC in a shaking water bath at 100 rpm for 2 hours. After gastric digestion, 2 mL of post-gastric sample was collected and frozen for further analyses.This sample was referred to as “POSTGASTRIC” sample. Remaining portion was used for the second

step of in vitro digestion process. Second step consisted of the simulation of intestinal digestion. For this purpose, remaining part from the post gastric sample was placed in a 250 mL glass beaker and 4.5 mL of 4 mg/mL pancreatin solution and 4.5 mL of 25 mg/mL of bile salt solution was added to beaker. The dialysis tube was used to simulate transition to blood in intestine. A part of the dialysis tubing was filled with sufficient amount of $NaHCO_3$ and placed in glass beaker. Then, the glass beaker was incubated again in the same conditions that was used for gastric digestion.

At the end of the method, the solution outside the dialysis tubing was taken as the "OUT" sample and the solution in the dialysis tubing was referred as the "IN" sample. It was presumed that, OUT corresponds to the portion remained in the gastrointestinal tract and IN corresponds to the portion passed to the serum. PG, IN and OUT samples were centrifuged at 18000 rpm for 15 min, where temperature was kept constat at 4ºC. Centrifuged samples were used for further analyses. All the results were corrected by measuring blank samples and subtracting the value of blank sample from final values of the samples.

Before HPLC analyses of samples from bioaccesibility assay, a seperation step was carried out to seperate phenolics from bile salts according to McDoughall *et al*. (2005) with some modifications. Samples were acidified to 0.5% (v/v) with trifloroacetic acid, mixed and centrifuged at 18000 rpm for 15 min. Then samples were filtered through a 0.45µm filter and analyzed.

3.4.7.HPLC profiles of the samples

For HPLC analyses, methanol extracts of the samples were used. Before injection of the samples, they were filtered through a 0.45µm filter. The HPLC system comprised of a Waters 600 control unit with a Waters 996 photodiode array (PDA) detector (U.S., and a column incubator at 25 °C). Column used for analyses was a C18 column (Luna®, U.S.A.) (150 mm x 4.60 mm i.d., 5 µm particle size, 100Aº pore size). Solvent systems were A (Ultra pure water with 0.1% trifluoroacetic acid (TFA) and B (acetonitrile with 0.1% TFA) for flavonoids with a flow rate of 1 mL/min. Separation of compounds in the extracts was conducted in a 60 min run.

3.5 Statistical Analysis

One-way ANOVA was used to investigate the remarkable differences between expected and observed values of samples ($p<0.05$) by using SPSS Statistics Software for Windows (Version 16.0, U.S.A.).

4. RESULTS AND DISCUSSION

Results of total phenolic, flavonoid, anthocyanin contents and four different antioxidant capacity assays were discussed for oat meal, blueberry *(Vaccinium arctostaphylos L.)*, whole milk and skimmed milk. In addition, the effect of milk addition was discussed for any significant difference. Total phenolic content of samples after in vitro digestion were compared to total phenolic content results obtained from acetone exracts of the samples.

4.1. Extraction of the Samples

Different solvent systems such as 70% ethanol, 80% methanol, methanol:acetone:water:acetic acid (40:40:29.5:0.5, v/v/v/v) and acetone:water (70:30, v/v) were tested in the extraction step. After evaluation of the results, acetone:water (70:30, v/v) solvent system was determined to have higher yield for extracting phenolic content than other solvents tested. As a result, acetone:water (70:30, v/v) solvent system was chosen as the solvent for the first part of the research. For HPLC analysis, methanol was chosen because of the fact that it was one of the most common solvents and most effective solvent in anthocyanin determination (Ignat *et al*., 2011). The extracts were prepared with 75% methanol:water (75:25, v/v) including 0.1% formic acid.

4.2. Total Phenolic, Flavonoid, Anthocyanin Content Analyses

Total phenolic and anthocyanin contents of samples were determined by using Folin method and pH differential method respectively. Total flavonoid content of samples were also assessed.

4.2.1.Total phenolic content

The total phenolic contents of oat meal and blueberry *(Vaccinium arctostaphylos L.)* were determined. In addition, whole milk and skimmed milk samples were also

analyzed to determine the additional effects coming from these ingredients in the food matrices. Results of total phenolic contents are given in Table 4.1.

Table 4.1 : Total phenolic contents of the samples.

Samples	Total Phenolic Content (mg GAE/ g fresh weight).
O	1.03±0.04
B	12.43±0.34
M1	0.53±0.02
M2	0.50±0.01
OM1	0.64±0.03
OM2	1.62±0.04
BM1	1.04±0.05
BM2	0.94±0.04
OB	2.90±0.09
OBM1	1.21±0.02
OBM2	1.15±0.02

Data were expressed as mean value±SD.

As can be seen from Table 4.1, phenolic content of oat meal was 1.03±0,04 mg GAE/g fresh sample which was found to be lower than the results of Ryan *et al*. (2011) who compared the phenolic contents of various oat-based breakfast cereals including different sources of polyphenols. They have found that the phenolic contents were varied in the range of 1.506-1.853 mg GAE/g fresh sample. On the other hand, Yu *et al*. (2002) have found lower values (0.505 mg GAE/g sample) as a result for total phenolic content of oat meal ethanol extracts.

Total phenolic content of blueberry *(Vaccinium arctostaphylos L.)* was found to be 12.43±0.34 mg GAE/g fresh sample which was higher than literature data. In fact, it was reported that there is a wide range of difference among cultivars and varities of the samples in the literature. However, our study was not conducted to detect the difference between cultivars and only one variety of wild blueberry sample was used for the preparation of breakfast cereals in the experiments. Koca and Karadeniz (2009) compared total phenolic content of different wild and cultivated blueberries grown in Black Sea Region of Turkey and found that wild blueberries had total phenolic content in the range of 3.08 - 5.42 mg GAE/g fresh sample. Ehlenfeldt and

Prior (2001) found that avarage total phenolic content of different blueberry cultivars was 1.79 mg GAE/g fresh sample. Moyer et.al (2002) used 70:30 acetone/water extract of blueberry samples to determine total phenolic contents of thirty different *Vaccinium* genotypes and found a wide range of total phenolic content 1.71-9.62mg GAE /g fresh sample. In a study carried out by Sellappan et. al (2002), total polyphenols of the Georgia grown blueberry samples were found to be between 2.62 to 9.30 mg GAE /g fresh sample. The differences in the total phenolic contents may be attributed to environmental and genetic factors of samples as discussed before by Kalt et. al (2001).

The phenolic content of whole milk and skimmed milk were also determined to assess the contribution of milk samples to phenolic contents of the blends. The results showed that both whole milk and skim milk gave positive response to phenolic assay. Whole milk and skimmed milk were found to have 0.53 ±0.02 and 0.50±0.01 mg GAE /g fresh sample, respectively. This effect may be attributed to proteins such as caseins or whey proteins in milk acting as polyphenols in total phenolic assay (Tong *et al*., 2000; Rival et. al, 2001; Park Y., 2009).

4.2.2.Total flavonoid content

The total flavonoid contents of oat meal and blueberry *(Vaccinium arctostaphylos L.)* were determined. Whole milk and skimmed milk samples were also analyzed to determine the additional effect coming from these ingredients. The results were expressed as mg catechin equivalent/g fresh sample (mg CE/g fresh weight. Blueberry, oat meal, whole milk and skimmed milk samples were found to have 4.67 ±0.12; 0.36 ±0.03; 0.22 ±0.01; 0.18 ±0.00 mg catechin equivalent/g fresh sample (mg CE/g fw) respectively.

Flavonoid content of blueberry was in an agreement with Sellapan *et al*. (2002), indicating flavonoid content in blueberry. The results of other ingredients such as oat and milk were lower than blueberry as expected due to the fact oat was not a sufficient flavonoid source as well as other two milk samples. The flavonoid content of OB sample was found to be 1.27±0.03 mg catechin equivalent/g fresh sample. The values of blends were low due to the fact that they can be thought as dilutions in milk. The results of flavonoid content analysis are given in Table 4.2.

Table 4.2 : Total flavonoid contents of the samples.

Samples	Total Flavonoid Content (mg CE/ g fresh weight).
O	0.36±0.03
B	4.68±0.12
M1	0.22±0.01
M2	0.18±0.00
OM1	0.25±0.00
OM2	0.23±0.01
BM1	0.46±0.02
BM2	0.33±0.01
OB	1.27±0.03
OBM1	0.45±0.01
OBM2	0.43±0.01

Data were expressed as mean values±SD.

4.2.3.Total anthocyanin content

The results of anthocyanin content analysis are given in Table 4.3.

Table 4.3 : Total anthocyanin contents of the samples.

Samples	Total Anthocyanin Content (mg C3GE/ g fresh weight).
O	0.00
B	2.51±0.04
M1	0.00
M2	0.00
OM1	0.00
OM2	0.00
BM1	0.14±0.07
BM2	0.10±0.02
OB	0.71±0.02
OBM1	0.20±0.01
OBM2	0.19±0.01

Data were expressed as mean ±SD.

The results were expressed as mg cyanidin 3-glucoside equivalents (C3GE) / g fresh weight. The results of total anthocyanin content analysis of showed that blueberry is a good source of anthocyanins having a content of 2.51±0.04 mg cyanidin 3-glucoside equivalents (C3GE) / g fresh weight. However, the values obtained from oat meal was very low (0.008±0.005 mg C3GE/ g fresh weight) as expected. For this reason, the anthocyanin content of oat meal was accepted as zero. Total anthocyanin content assay was also performed for whole and skimmed milk for further analysis to decide if there is a contribution of whole and skimmed milks in the food matrices. Both of milk samples had no anthocyanin content as expected. To determine the effect of milk on OBM1 and OBM2 samples, the anthocyanin content of OB sample was analyzed and found to be 0.71±0.02 mg C3GE/ g fresh weight.

Anthocyanin content of blueberries *(Vaccinium arctostaphylos L.)* were analyzed by different researchers. Our finding was higher than some previous studies (Ehlenfeldt and Prior, 2001; Sellappan *et al*., 2002) and lower than a study conducted by Moyer et.al. (2002). On the other hand, there was an agreement with a study conducted by Koca and Karadeniz (2009) investigating the total anthocyanin contents of blueberries grown in Black Sea Region of Turkey (0.18-2.94 mg C3GE/ g fresh weight). Moze *et al*. (2011) analyzed the total anthocyanin contents of blueberries from one location in Slovenia and anthocyanin content was 2.12 ±0.14 mg C3GE/ g fresh weight and this value was lower then our finding. The values of blends were low due to the fact that they can be thought as dilutions in milk.

4.3 Antioxidant Capacity Assays

Four different antioxidant capacity assays were performed to determine the antioxidant capacity of the ingredients and the blends. Using various antioxidant capacity assays was recommended and would be an asset to determine the the antioxidant activity in a correct manner (Aruoma, 2003).

4.3.1 ABTS assay

The results of ABTS assay were expressed as μmol trolox equivalent /g fresh weight (μmol TE/g fresh weight), data are shown in Table 4.4.

Table 4.4 : The TAA of the samples according to ABTS assay.

Samples	Total Antioxidant Capacity (μmol TE/ g fresh weight).
O	3.42±0.03
B	85.06±1.14
M1	1.35±0.02
M2	1.90±0.09
OM1	2.06±0.03
OM2	2.51±0.09
BM1	6.18±0.27
BM2	5.89±0.44
OB	23.62±0.73
OBM1	6.90±0.06
OBM2	7.30±0.04

Data were expressed as mean values±SD.

Oat meal and blueberry had 3.42±0.03; 85.06±1.14 μmol TE/g fresh weight respectively. Blend of oat meal and blueberry sample (OB) was also analyzed in order to assess the effect of milk on OBM1 and OBM2 blends. Antioxidant capacity of OB sample was found to be 23.62± 0.73 μmol TE/g fresh weight. The antioxidant capacity of whole milk was found to be 1.35±0.02 μmol TE/g fresh weight which was lower than skimmed milk having 1.900±0.09μmol TE/g fresh weight. This finding was higher than 0.6±0.1μmol TEAC value which was reported by Dubeau *et al*. (2010). Our findings showed contrary results to the study carried by Chen *et al*. (2003). In the previous study, whole milk was found to have higher antioxidant capacity than skimmed milk and the antioxidant capacity of milk was found to be higher due to increasing fat content when analysing with ABTS method. This effect may be sourced from differences in the ABTS method between the previous study and our recent study.

4.3.2 CUPRAC assay

The results of CUPRAC assay were expressed as μmol trolox equivalent /g fresh weight (μmol TE/g fresh weight) and shown in Table 4.5.

Table 4.5 : The TAA of the samples according to CUPRAC assay.

Samples	Total Antioxidant Capacity (µmol TE/ g fresh weight).
O	24.74±1.06
B	321.49±2.80
M1	9.99±0.19
M2	9.31±0.91
OM1	12.66±1.02
OM2	12.44±0.20
BM1	26.41±0.73
BM2	23.86±0.23
OB	71.79±5.61
OBM1	50.54±1.24
OBM2	46.46±2.26

Data were expressed as mean values ±SD.

The CUPRAC assay was carried out for blueberry, oat meal, whole milk and skimmed milk and the antioxidant capacities were 321.49±2.80; 24.74±1.06; 9.99±0.19; 9.31±0.91 µmol TE/g fw respectively. Whole milk showed higher antioxidant capacity than skimmed milk. OB sample was analyzed and the results was used to assess the effect of milk on OBM1 and OBM2 samples. OB sample was found to have 71.79±5.61 µmol TE/g fw. It was observed that CUPRAC method was concluded with the highest antioxidant capacity for all of the samples.

4.3.3 DPPH assay

The results of DPPH assay were expressed as µmol trolox equivalent /g fresh weight (µmol TE/g fresh weight) and are shown in Table 4.6. The DPPH radical was used to investigate the antioxidant capacity of the individual ingredients and their blends.

Table 4.6 : The TAA of the samples according to DPPH assay.

Samples	Total Antioxidant Capacity (µmol TE/ g fresh weight).
O	1.74±0.01
B	40.47±0.26
M1	0.00
M2	0.00
OM1	0.54±0.13
OM2	0.57±0.07
BM1	3.68±0.01
BM2	3.81±0.17
OB	14.76±0.52
OBM1	4.28±0.02
OBM2	3.96±0.06

Data are expressed as mean values ±SD.

The results showed that blueberry had antioxidant capacity equal to 40.47±0.26 µmol trolox equivalent/ g fresh sample (µmol TE/g fw). Antioxidant capacity of oat meal was 1.74±0.01µmol TE/g fw. However, the results of whole milk and skimmed milk were not reasonable since they had higher absorbance values than blank samples so that both mik samples were accepted to have no antioxidant capacity according to DPPH assay. OB sample was found to have a 14.76±0.52µmol TE/g fw antioxidant capacity.

4.3.4 FRAP assay

The results of FRAP assay were expressed as µmol trolox equivalent /g fresh weight (µmol TE/g fresh weight) and are shown in Table 4.7. The results indicated that blueberry had the highest antioxidant capacity which was 34.97 ±1.15 µmol TE/g fresh weight in accordance with literature. The FRAP value were found to vary between 7.41-57.92 µmol TE/g fresh weight for wild and cultivated blueberry samples by Koca and Karadeniz (2008).

Table 4.7 : The TAA of the samples according to FRAP assay.

Samples	Total Antioxidant Capacity (µmol TE/ g fresh weight).
O	2.40±0.07
B	34.97±1.15
M1	0.54±0.04
M2	0.09±0.01
OM1	0.98±0.03
OM2	0.89±0.03
BM1	3.01±0.01
BM2	2.82±0.03
OB	9.74±1.24
OBM1	2.91±0.08
OBM2	3.00±0.02

Data are expressed as mean values ±SD.

Oat meal was found to have 2.39±0.07 µmol TE/g fresh weight. Ryan et. al (2011) analyzed thirty different oat based breakfast cereals to assess their antioxidant capacities. They ranged between 1682–3542 mmol/l FRAP. Whole milk showed higher antioxidant capacity (0.54±0.04µmol TE/g fresh weight) than skimmed milk (0.09±0.01µmol TE/g fresh weight). This finding was agreement with the study carried out by Chen *et al*. (2003) by contrast with ABTS method. The antioxidant capacity of OB sample was measure to be 9.74±1.24 µmol TE/g fresh weight.

4.4. Changes in Phenolic Contents and Antioxidant Potentials of Food Matrices.

With the aim of detecting the changes of phenolic contents in food mixtures, expected values were calculated mathematically based on the ratio among the ingredients in the mixture (oat meal:blueberry:milk 4:1:8 w/w/w) by summing the seperate effects of ingredients except for oat meal+blueberry+milk combinations (OBM1, OBM2).

To evaluate the expected values for OBM1 and OBM2 samples, oat meal+blueberry sample (OB) results were used instead of seperate oat meal and blueberry (O and B)

values. The phenolic contents of each ingredient was multipled by its ratio in the mixture and then divided to total ratio for calculating expected values.

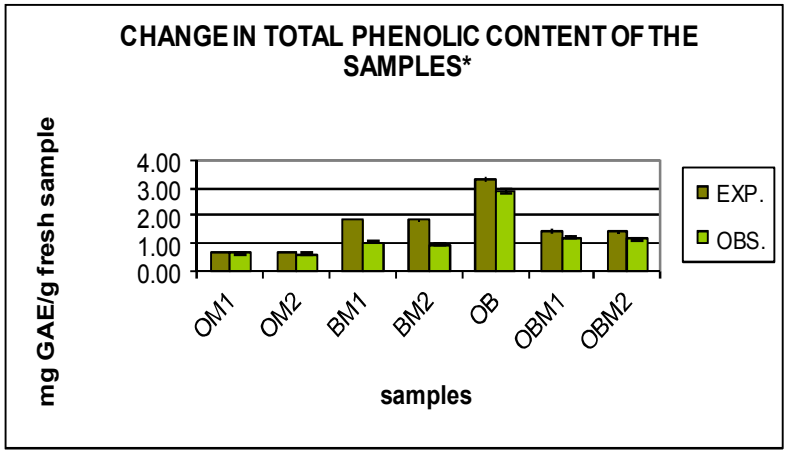
Statistical analyses were applied to the results in order to compare the differences between expected and observed values (p<0,05). If there was no significant difference between expected and observed value, it would indicate an "additive interaction". If observed values were found to be higher than expected values with statistical difference, it would express a "synergistic interaction". On the contrary, if observed values were found to be lower than expected values with a statistical difference, it would indicate an "inhibition effect". The results are given in Table 4.8 and Table 4.9.

Table 4.8 : The change in TPC , TFC and TAC values of samples.

Samples		TPC (mgGAE/g fw)		TFC (mg CE/ g fw)		TAC (mg C3GE/g fw)	
OM1	E	0.70±0.00		0.27±0.01			
			(In)		(In)		
	O	0.64±0.03		0.25±0.00		ND	
OM2	E	0.68±0.02		0.24±0.01			
			(Ad)		(Ad)		
	O	0.62±0.04		0.23±0.01		ND	
BM1	E	1.85±0.02		0.72±0.02		0.28±0.00	
			(In)		(In)		(In)
	O	1.04±0.05		0.46±0.02		0.14±0.07	
BM2	E	1.82±0.05		0.68±0.01		0.28±0.00	
			(In)		(In)		(In)
	O	0.94±0.04		0.33±0.01		0.10±0.02	
OB	E	3.31±0.10		1.23±0.02		0.50±0.01	
			(In)		(Ad)		(Sy)
	O	2.90±0.09		1.27±0.03		0.71±0.02	
OBM1	E	1.44±0.05		0.63±0.01		0.27±0.01	
			(In)		(In)		(In)
	O	1.21±0.02		0.45±0.01		0.20±0.01	
OBM2	E	1.42±0.03		0.60±0.01		0.27±0.01	
			(In)		(In)		(In)
	O	1.15±0.02		0.43±0.01		0.19±0.01	

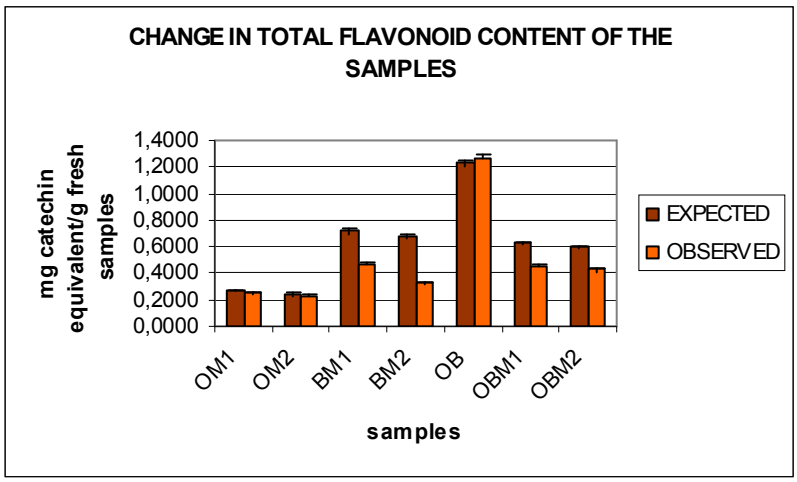
Data are expressed as mean values ±SD. Datas examined for a significant difference between O and E values (p<0,05).O, observed value; E, expected value; Sy, synergistic effect; Ad, additive effect; In, inhibition effect. ND means not detected.

Each combination of oat meal, blueberry, whole and skimmed milk were evaluated for all assays mentioned in the study and the results are illustrated in Figure 4.1, Figure 4.2, Figure 4.3. The results of total anthocyanin content for OM1 and OM2 samples were evaluated as zero due to seperate results of milk and oat meal samples possesing no anthocyanin content. As a result, the values are shown as not detected (ND) in the tables.



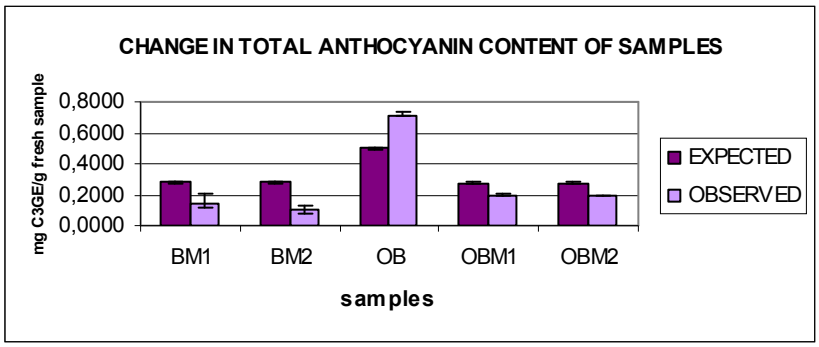
*The results were expressed as mg GAE/g fresh sample.

Figure 4.1 : Change in total phenolic content of the samples.



*The results were expressed as mg CE/g fresh sample.

Figure 4.2 : Change in total flavonoid content of the samples.



*The results were expressed as mg C3GE/ g fresh sample.

Figure 4.3 : Change in total antocyanin content of the samples.

The results indicated that combinations with milk generally resulted in inhibition on total phenolic, flavonoid and anthocyanin contents. The results from combination of oat meal, whole milk and skimmed milk (OM1, OM2 samples) revealed that whole milk had an inhibition effect on total phenolic and flavonoid contents. However, skimmed milk had only an additive effect on these values.

The study carried out by Sharma *et al*. (2008) reported that milk addition to tea extracts resulted in a significant decrease in total phenolic content of tea samples and this effect was thought to be due to the covalent or non covalent interactions between plant phenolics and milk proteins. This effect was reported to be a masking effect of milk proteins (caseins, whey proteins) on polyphenols like catechins and flavonoids.

The results from interactions of blueberry, whole milk and skimmed milk also resulted in a significant decrease in total phenolic, flavonoid and anthocyanin contents of samples so they were reported as an inhibition effect. OBM1 and OBM2 samples were found to have lower phenolic contents than expected. Whole milk and skimmed milk had inhibition effect on their phenolic content. These findings were agreement with studies in which binding interactions between polyphenols such as flavanoids and milk proteins were reported (Yüksel *et al*., 2010; Hasni *et al*., 2011).

The observed results of OB samples were found to be significantly lower than expected values for TPC assay. Wang *et al*. (2011) reported that total phenolic content of food mixtures may be modified by antogonistic interactions between components. For TAC assay, OB sample was found to have a higher value than expected value, that effect may be related with potential interactions among phenolics reported previously by Salucci *et al*. (1999).

Results showed that whole milk and skimmed milk had different degrees of inhibition effects on total phenolic content. For oat meal samples, adding skimmed milk had no significant effect on phenolic content of sample unlike whole milk. The effect of whole milk and skimmed milk was given as percentages in Figure 4.4.

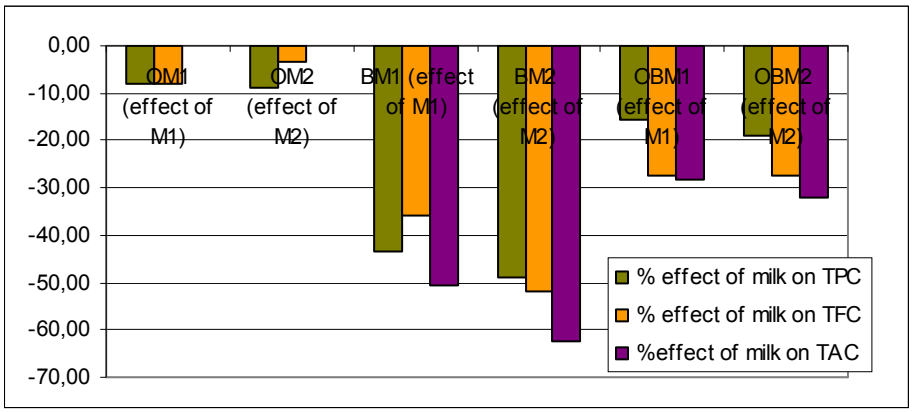


Figure 4.4 : The effect of whole and skimmed milk on TPC, TFC and TAC values of samples as percentages.

Whole milk had 7.91%, 43.60% and 15.40% inhibition effects on total phenolic content of oat meal, blueberry and oat meal+blueberry samples, respectively. The inhibition effect was 8.7% , 35.64% and 27.53% for total flavanoid content of samples in the same order.

According to total anthocyanin content results, 50.77% and 28.40% inhibition effect was observed for B and OB samples. According to skimmed milk results, it had a negative effect causing decrease such as %9.07, 48.72% and 19.10% on TPC values of O, B and OB samples respectively.

The TFC showed similar results. Skimmed milk diminished the TFC of O, B and OB samples at 3.24%, 51.73% and 27.54%, respectively. For TAC assay results, it caused 62.36% and 31.86% decrease in values of B and OB samples.

As can be seen form the Figure 4.4, skimmed milk was found to show higher inhibiton percentages than whole milk. Although skimmed milk had a higher inhibition percentage on total phenolic content on oat meal sample (O), statistical analyses showed that there was no significant difference between expected and observed values for OM2 sample so its effect was regarded as an additive effect in Table 4.8.

Oat meal sample was accepted as possesing no anthocyanin content due to its low antocyanin content mentioned before so that the effect of milk on OM1 and OM2 samples are not shown in Figure 4.4.

The previous study carried out by Serafini *et al*. (2009) reported that addition of milk on blueberry extracts caused decrease on phenolic content of samples. This finding was in agreement with our study. However, in this research done by Serafini *et al*. (2009), whole milk was reported to be more effective than skimmed and semi-skimmed milk but the results obtained from our study were not in agrement with that study.

On the other hand, there were also studies reporting that skimmed milk had higher diminishing effects than whole milk (Krul *et al*., 2001; Ryan *et al*., 2010). Ryan *et al.* (2010) studied the effect of 10, 15 and 20 mL bovine milk addition on tea antioxidant capacity. FRAP method was used to determine the antioxidant capacity of samples. Three types of milk (whole milk, semi-skimmed milk and skimmed milk) were used in study. The results showed that the addition of semi-skimmed milk and skimmed milk decreased total antioxidant capacity significantly when compared to water addition in same amaunt to tea and whole milk showed a lower effect on total antioxidant capacity compared to semi-skimmed milk and skimmed milk.

Statistical analyses were also applied to the results of antioxidant capacity assays in order to compare the differences between expected and observed values (p<0,05). If there was no significant difference between expected and observed value, it would indicate an “additive interaction”. If observed values were found to be higher than expected values with statistical difference, it would express a “synergistic interaction”. On the contrary, if observed values were found to be lower than expected values with a statistical difference, it would indicate an “inhibition effect”.

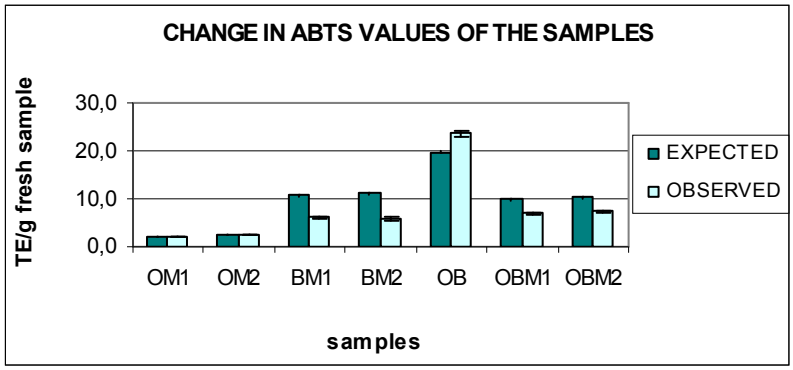
The effect of blending ingredients on ABTS, CUPRAC, DPPH and FRAP values of samples are given in Table 4.9.

Table 4.9 : The change in ABTS, CUPRAC, DPPH and FRAP values of blends.

Samples		ABTS (µmol TE/g fw)		CUPRAC (µmol TE/g fw)		DPPH (µmol TE/g fw)		FRAP (µmol TE/g fw)	
OM1	E	2.04±0.01		14.91±0.43		0.58±0.00		1.16±0.03	
	O	2.06±0.03	Ad	12.66±1.02	In	0.54±0.13	Ad	0.98±0.03	In
OM2	E	2.40±0.05		14.45±0.59		0.58±0.00		0.86±0.03	
	O	2.51±0.09	Ad	12.44±0.20	In	0.57±0.07	Ad	0.89±0.03	Ad
BM1	E	10.65±0.11		44.60±0.40		4.50±0.03		4.37±0.15	
	O	6.18±0.27	In	26.40±0.73	In	3.68±0.01	In	3.01±0.01	In
BM2	E	11.14±0.11		43.50±1.11		4.50±0.03		3.97±0.14	
	O	5.89±0.44	In	23.86±0.23	In	3.81±0.17	In	2.82±0.03	In
OB	E	19.74±0.25		84.09±0.67		9.48±0.04		8.91±0.27	
	O	23.62±0.73	Sy	71.79±5.61	In	14.76±0.52	Sy	9.74±1.24	Ad
OBM1	E	9.92±0.26		33.76±2.05		5.68±0.20		4.08±0.50	
	O	6.90±0.06	In	50.54±1.24	Sy	4.28±0.02	In	2.91±0.08	In
OBM2	E	10.25±0.24		33.34±2.06		5.68±0.20		3.81±0.48	
	O	7.30±0.04	In	46.46±2.26	Sy	3.96±0.06	In	3.00±0.02	In

Datas are expressed as mean values±SD. Datas examined for a significant difference between O and E values ($p<0,05$).O, observed value; E, expected value;Sy, synergistic effect; Ad, additive effect; In, inhibition.

Four different antioxidant capacity assays were carried out in the study and differences were observed among these assays. This difference may be related to different phenolic profile of blueberry and oat samples, potential interactions between their ingredients and milk (Yüksel *et al*., 2010; Hasni *et al*., 2011; Wang *et al*., 2011).

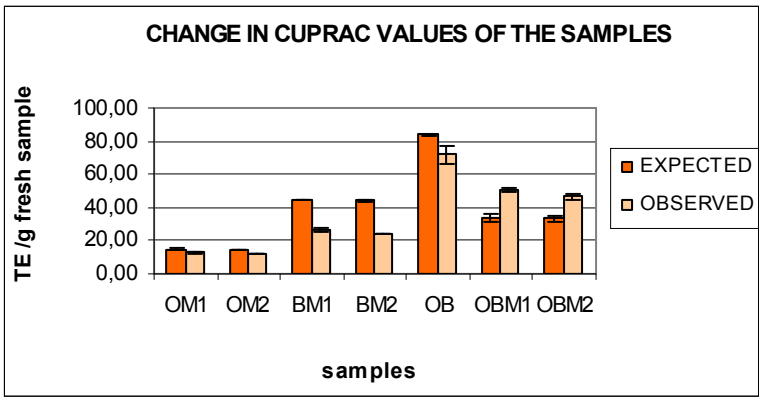


*Results were expressed as µmol TE /g fresh sample.

Figure 4.5 : Change in ABTS values of the samples.

Addition of milk had different effects on antioxidant capacities of the samples that were analyzed by ABTS method. For instance, whole milk addition on oat sample resulted in an additive effect. For B and OB samples, milk addition resulted in inhibition effect. Dubeau *et al*. (2010) studied the effect of 2% skimmed milk on tea samples by using ABTS method. The results indicated that milk caused no significant decrease in antioxidant capacity. In their study, milk percentage in the sample was was very low (5%) compared with that of our study. The difference may be explained by different ratios of milk addition and different phenolic profile of blueberry and oat samples and tea.

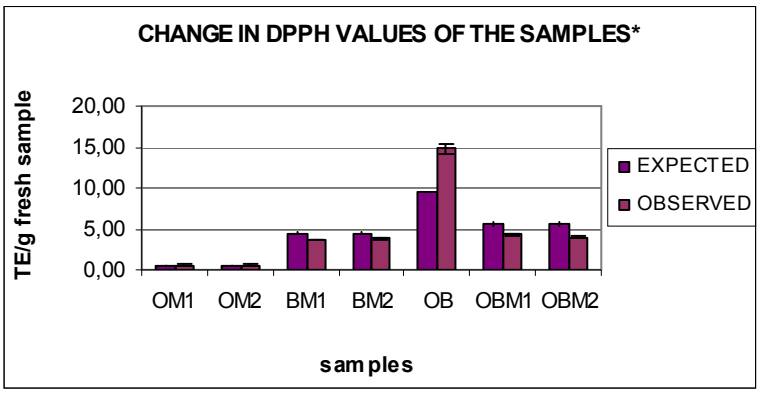
The synergistic effect was observed for OB samples. This effect can be explained by potential interactions between blueberry and oat phenolics. Wang (2011) reported that combining specific food across different categories were more likely to cause synergistic interaction.



*Results were expressed as µmol TE /g fresh sample.

Figure 4.6 : Change in CUPRAC values of the samples.

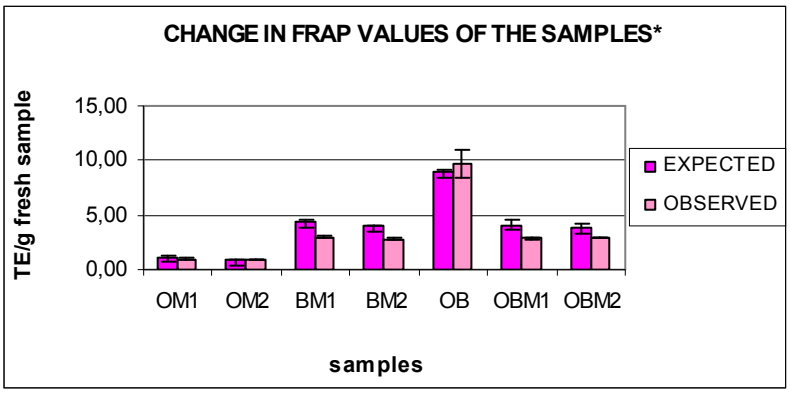
CUPRAC results were not agrement with ABTS results for OM1 and OM2 samples and siginificant decrease was only observed for OM1, OM2, BM1 and BM2 samples. On contrary, there was a synergistic effect of whole and skimmed milk on OB sample. Mixing oat meal and blueberry resulted in an inhibition effect. The differences on results between methods was also previously reported by Wang *et al*. (2011).



*Results were expressed as µmol TE /g fresh sample.

Figure 4.7 : Change in DPPH values of the samples.

The results of DPPH assay was totally agreement with ABTS assay results. Additive effect for OM1 and OM2 samples, inhibition effect for BM1, BM2, OBM1 and OBM2 samples were observed. The synergistic effect of combining oat and blueberry was also observed due to potential interactions among phenolics (Wang *et al*., 2011).



*Results were expressed as µmol TE /g fresh sample.

Figure 4.8 : Change in FRAP values of the samples.

For oat samples, whole milk had an inhibition effect. However, skimmed milk had no significant effect. In addition, two types of milk had negative influence on total antioxidant capacity of BM1, BM2, OBM1 and OBM2 samples. The milk addition to blueberry sample was also studied by Serafini *et al*. (2009) and resulted in similar results. Addition of milk to blueberry resulted in decrease in FRAP values of samples.

It was posssible to see differences among the results of antioxidant capacity assays. For this reason, it was recommended to use different antioxidant capacity assays in asssesing the antioxidant capacity of foods in a true manner (Skrede *et al*., 2004; Çapanoğlu *et al*., 2008; Albayrak *et al*, 2010).

To investigate the relation between total phenolic, flavonoid, anthocyanin content and antioxidant capacity assays, the regression cofficients were estimated. Positive relations were observed between total phenolic, flavonoid and anthocyanin assay and four antioxidant capacity assays for the samples. The regression coefficient values (R^2) are given in Table 4.10 and regression graphics are presented in Appendix B.

Table 4.10 : The corelation coefficients between TPC,TFC,TAC and AOX capacity assays.

	Antioxidant Capacity Assays			
Assay	ABTS	CUPRAC	DPPH	FRAP
TPC	0.9948	0.9916	0.9719	0.9946
TFC	0.9981	0.9879	0.9837	0.9819
TAC	0.9987	0.9847	0.9927	0.9958

These values indicated that antioxidant capacity was strongly related with total phenolic, flavanoid and anthocyanin contents.

4.5 HPLC Profiles of the Samples

Seperation of phenolic acids, flavanoids and anthocyanins were carried out by using HPLC system. Phenolic composition of oat meal and blueberry samples are given in Table 13. Some of the phenolics were not detected in samples so that they were expressed as ND (not detected) in the Table 4.11.

Table 4. 11 : Phenolic Composition of oat meal and blueberry samples.

Phenolic Compounds	Phenolic Composition mg/ 100g fresh weight	
	Blueberry *(Vaccinium arctostaphylos L.)*	Oat Meal
Caffeic acid	ND	0.47±0.13
Chlorogenic acid	177.10±22.9	ND
p-Coumaric acid	0.68±0.05	ND
Ferulic acid	ND	0.45±0.10
Gallic acid	ND	3.15±0.83
Total Phenolic Acids	177.78	4.07
Catechin	ND	1.50±0.00
Quercetin-3-galactoside	2.28±0.00	ND
Quercetin-3-ß-d-glucoside	0.57±0.00	ND
Total flavonols	2.85	
Cyanidin chloride	0.69±0.00	
Cyanidin 3-o glucoside	7.59±0.54	
Cyanidin-3-o-rutinoside chloride	4.03±1.25	
Delphinidine chloride	18.28±0.00	
Delphinidin 3-glucoside	30.61±1.64	
Malvidin-3-galactoside	5.40±0.2	
Pelargonin Chloride	68.53±4.6	
Pelargonidin-3-O-glucoside	16.20±0.00	
Peonidin-3-glucoside	1.41±0.00	
Petunidin chloride	7.00±0.00	
Total Anthocyanins	159.47	

The results were expressed as mean values ± SD. ND: not detected.

Five phenolic acids were detected for oat meal and blueberry samples. Caffeic, ferulic and gallic acid were found in oat meal samples and chlorogenic and p-coumaric acid were found in blueberry samples. On contrary to the study carried out

by Serafini *et al*. (2009) reporting the presence of ferulic acid and gallic acid in blueberry samples, ferulic acid and gallic acid were not detected in the blueberry samples used in our study. According to Serafini *et al*. (2009), the amount of chlorogenic acid was 24.19 mg /100g sample. However, our findings were much higher (177.10±22.9 mg/100g fresh sample). The difference might be sourced from different exraction solvents used in the studies.

Catechin was detected in oat meal samples. However, it was not found in blueberry samples contrary to a previous study reporting catechin as the mojor flavonoid in blueberry samples (Sellappan *et al*., 2002). This difference might be attributed to different extraction method in which 4% acetic acid in acetonitrile was selected as the extraction solvent in the study or might be sourced from a wide range of phenolic composition among cultivars and varities of the blueberry samples (Sellappan *et al*., 2002; Moyer et. al., 2002; Cho *et al*., 2005).

Quercetin-3-galactoside and quercetin-3-ß-d-glucoside was the detected flavonols in the blueberry samples. The amount of quercetin-3-galactoside and quercetin-3-ß-d-glucoside were lower than the results obtained by Cho *et al* (2005) and higher than a previous study conducted by Serafini *et al*. (2009). The quercetin-3-galactoside were found to be higher than quercetin-3-ß-d-glucoside in agreement with previous studies (Cho *et al*., 2005; Serafini *et al*., 2009).

According to Lättı *et al*. (2009), the average total anthocyanin content in Caucasian blueberries native to Turkey was 1420 mg/ 100g dry weight. We observed 159.47 mg/100g fresh weight which was equal to 1138.61 mg/dry weight. Our finding was relatively lower because of the fact that the previous study studied different Caucasian blueberries from different locations.

The results of phenolic profile showed that blueberry had a wide range of anthocyanin composition as mentioned before by several researchers (Sellapan *et al*., 2002; Lättı *et al*., 2009; Koca and Karadeniz, 2009; Serafini *et al*., 2009).

Delphinidine 3-glucoside, cyanidin glucoside and peonidin-3-glucoside contents were found to be 30.61±1.64, 7.59±0.54 and 1.41±0.00mg/100g fw respectively in blueberry samples. These findings were lower than the previous study by Lattı *et al*. (2009). On the other hand, malvidin-3-galactoside content of our blueberry sample was 5.4±0.2mg/100g fresh weight which was higher. Delphinidine 3-glucoside,

cyanidin 3-o-glucoside, peonidin-3-glucoside contents were found to be higher than the findings of Serafini *et al*. (2009). However, malvidin-3-galactoside content of our samples was lower than this study.

Combinations of oat meal, blueberry, whole milk and skimmed milk were also analyzed for their phenolic profile by HPLC. OM1 and OM2 samples were accepted as dilutions of O sample in milk due to the fact that whole and skimmed milk samples were found to have no phenolic content. The results were shown in Table 4.12. Dilution factor was 3 for OM1 and OM2 samples by summing up oat ratio (1) and milk ratio (2) in mixtures.

Table 4.12 : Phenolic acid and catechin profile of O, OM1 and OM2 samples.

Samples	Phenolic Compound Composition mg/100g fresh weight			
	Caffeic acid	Ferulic acid	Gallic acid	Catechin
O	0.47±0.13	0.45±0.10	3.15±0.83	1.50±0.00
OM1	0.21±0.06	0.14±0.00	1.29±0.41	ND
OM2	0.17±0.01	0.15±0.01	1.14±0.66	ND

The results were expressed as mean values± SD. ND: Not detected.

There was no decrease in gallic acid and caffeic acid contents of OM1 and OM2 compared to O sample. There was no difference in amount of ferulic acid in OM2 sample. However, OM1 sample was found to have lower ferulic acid content compared with expected value (6.67% lower). Although catechin had been detected in oat samples, it was not found in OM1 and OM2 samples. This finding was in agreemet with previous studies reporting the binding interactions between flavonoids and milk proteins (Yüksel *et al*., 2010; Hasni *et al*., 2011).

BM1 and BM2 samples also accepted as dilutions of B sample because of the fact that no phenolics were detected milk samples. Dilution factor was 9 for BM1 and BM2 samples by summing up blueberry ratio (1) and milk ratio (8) in mixtures. These results are shown in Table 4.13.

Table 4.13 : Phenolic acid and flavonol profile of B, BM1 and BM2 samples.

Samples	Phenolic Compound Composition mg/ 100g fresh weight				
	Chlorogenic acid	p-Coumaric acid	Caffeic acid	Quercetin-3-galactoside	Quercetin-3-ß-d-glucoside
B	177.10±22.9	0.68±0.05	ND	2.28±0.00	0.57±0.00
BM1	17.74±1.06	ND	ND	ND	ND
BM2	16.48±0.08	ND	ND	ND	ND

The results were expressed as mean ± SD. ND means not detected.

For instance, quercetin-3-galactoside and quercetin-3-ß-d-glucoside were not detected in the samples. It might be an effect of dilution or might be sourced from the potential interactions between quercetin and milk proteins as reported before by Galleano *et al*., (2010). On the other hand, chlorogenic acid was found to be slightly lower than expected values. Observed value of chlorogenic acid in BM1, BM2 samples were 9.8% and 16.2% lower than expected values, respectively. Anthocyanin profile of BM1 and BM2 are shown in Table 4.14.

Table 4.14 : HPLC anthocyanin profile of B, BM1 and BM2 samples.

Anthocyanins	Phenolic Compound Composition mg/ 100g fresh weight		
	B	BM1	BM2
Cyanidin chloride	0.69±0.00	ND	ND
Cyanidin 3-o glucoside	7.59±0.54	0.45±0.05	0.51±0.07
Cyanidin-3-o-rutinoside chlor.	4.03±1.25	ND	ND
Delphinidine chloride	18.28±0.00	ND	ND
Delphinidin 3-glucoside	30.61±1.64	2.34±0.13	3.34±0.13
Malvidin-3-galactoside	5.4±0.2	5.41±0.24	ND
Pelargonin Chloride	68.53±4.6	3.33±1.07	4.26±0.12
Pelargonidin-3-O-glucoside	16.20±0.00	ND	ND
Peonidin-3-glucoside	1.41±0.00	ND	ND
Petunidin chloride	7.00±0.00	ND	ND

The results were expressed as mean ± SD. ND means not detected.

Cyanidin 3-o-glucoside, delphinidin 3-glucoside and pelargonin chloride were lower than expected values in BM1 samples and results showed 46.6% 31.2% and 56.3% decrease, respectively. The results of BM2 sample were similar to BM1 but the decrease was 39.5% 2.0% and 44.0% for the quantities of cyanidin 3-o-glucoside, delphinidin 3- glucoside and pelargonin chloride, respectively.

For OB sample, there was no difference in chlorogenic acid content comparing to expected value. Catechin, caffeic acid and p-coumaric acid were not detected in OB sample, it might be sourced from dilution effect. On the other hand, there was an increase in anthocyanin contents such as cyanidin 3-o glucoside, cyanidin-3-o-rutinoside, delphinidin 3 -glucoside and pelargonin chloride and phenolic acid

contents of ferulic acid and gallic acid compared to expected values. These results are shown in Table 4.15 and Table 4.16 with the results of OBM1 and OBM2 samples.

Table 4.15 : Phenolic acid and catechin profile of OB, OBM1 and OBM2 samples.

Samples	Phenolic Compound Composition mg/ 100g fresh weight					
	Chlorogenic acid	p-Coumaric acid	Caffeic acid	Gallic acid	Ferulic acid	Catechin
OB	36.66±1.07	ND	ND	2.39±0.13	0.31±0.00	ND
OBM1	12.99±0.28	ND	ND	1.33±0.29	ND	ND
OBM2	12.18±2.31	ND	ND	1.52±0.32	ND	ND

The results were expressed as mean values± SD. ND: Not detected.

The results from OBM1 and OBM2 samples were based on OB HPLC results and any affect of milk was investigated. Dilution factor was 13/5 for OBM1 and OBM2 samples by summing up blueberry+oat ratio (5) and milk ratio (8) in mixtures and dividing it to blueberry+oat ratio (5). According to results of phenolic acids, catechin, caffeic acid and *p*-cuomaric acid and ferulic acid were not detected in OBM1 and OBM2 samples. There were 7.9% and 13.6% decrease in chlorogenic acid content of OBM1 and OBM2 samples compared to OB sample respectively. There was no decrease in gallic acid composition compared in OBM1 and OBM2 compared to OB samples.

Cyanidin chloride, delphinidin chloride, pelargonin-3-o-glucoside and peonidin-3-glucoside were not detected in samples because of the fact that these anthocyanins were also not detected in OB sample. Petunidin chloride was detected in OB sample but not in OBM1 and OBM2 samples. Cyanidin 3-o-glucoside and cyanidin -3-rutinoside contents were found to be decreased due to milk addition. The decrease in cyanidin-3-o-glucoside content was 21.1% and 17.2% for whole milk and skimmed milk, respectively. No decrease was detected for whole milk addition in cyanidin-3-rutinoside content.

Table 4.16 : HPLC anthocyanin profile of OB, OBM1 and OBM2 samples.

Anthocyanins	Phenolic Compound Composition mg/ 100g fresh weight		
	OB	OBM1	OBM2
Cyanidin chloride	ND	ND	ND
Cyanidin 3-o-glucoside	2.01±0.22	0.61±0.17	0.64±0.00
Cyanidin-3-o-rutinoside chloride	1.38±0.28	0.60±0.00	0.47±0.00
Delphinidine chloride	ND	ND	ND
Delphinidin 3-glucoside	16.85±0.35	2.75±0.28	2.26±0.10
Malvidin-3-galactoside	1.96±0.43	ND	0.48±0.04
Pelargonin Chloride	18.64±1.07	3.80±0.39	2.70±0.32
Pelargonidin-3-O-glucoside	ND	ND	ND
Peonidin-3-glucoside	ND	ND	ND
Petunidin chloride	2.40±0.00	ND	ND

The results were expressed as mean values± SD. ND: Not detected.

Decrease of cyanidin-3-rutinoside was 11.4% for skimmed milk addition. Delphinidin 3–glucoside had a lower value than expected and whole milk and skimmed milk addition resulted in 57.6% and 61.1% decrease, respectively. The results of pelargonidin chloride content was accordance with the results of delphinidin-3–glucoside content and resulted in 47.0% and 62.3% decrease respectively showing a higher reduction due to skimmed milk addition. Malvidin-3-galactoside was not detected in OBM1 sample. It was detected in OM2 sample with a lower content (36.3%) than expected value.

All these decreases reported in recent study needed to be explained by model systems searching for potential interactions between phenolics and milk proteins and also among phenolic composition. There was limited information in literature to express the potential interactions.

4.6.Bioaccessibility Assay

Bioaccessibility assay was performed by using an in vitro digestion procedure according to McDoughall *et al*. (2005). The procedure were applied for all samples and samples were analyzed for total phenolic content. Phenolic profiles were determined by HPLC .

4.6.1.Total phenolic content

Total phenolic content assay was performed for PG, IN and OUT samples of the individual ingredients such as oat meal, milk and blueberry samples as well as their blends. The results were compared with the total phenolic content results of acetone extracts of samples by accepting acetone exract values as control.The results are shown in Table 4.17 and Figure 4.9.

Table 4.17 : TPC recovery of samples after in vitro digestion procedure.

TPC Recovery as percentage according to control.				
Samples	Control Sample	PG Sample	IN Sample	OUT Sample
O	100	84.64±2.35	47.47±16.61	124.22±1.83
B	100	66.91±0.12	5.20±0.00	48.83±0.29
M1	100	149.03±5.72	62,43±31.47	161,18±4.81
M2	100	167.90±1.16	69.37±25.27	165.01±5.73
OM1	100	139.73±5.66	62.33±19.05	173.70±2.34
OM2	100	146.19±0.16	70.05±20.32	186.13±3.40
BM1	100	121.98±36.32	27.30±5.64	98.87±7.51
BM2	100	117.55±23.70	35.08±1.60	97.32±5.26
OB	100	30.01±8.00	15.63±3.10	40.37±4.58
OBM1	100	74.59±2.82	30.42±0.29	91.50±3.20
OBM2	100	79.90±2.49	32.10±0.13	99.54±3.69

Bioacessibility assay performed duplicate and analyses carried out triplicate.Results were shown as mean values±SD.

Although being in a wide range, the results showed that the recovery of phenolics from blueberry sample was low. Only 54% of total phenolic content could be recovered (sum of IN and OUT samples). These value was lower than the values in the literature reported for raspberry (McDoughall *et al*., 2005) and higher than values reported for red cabbage (McDoughall *et al*. 2007).

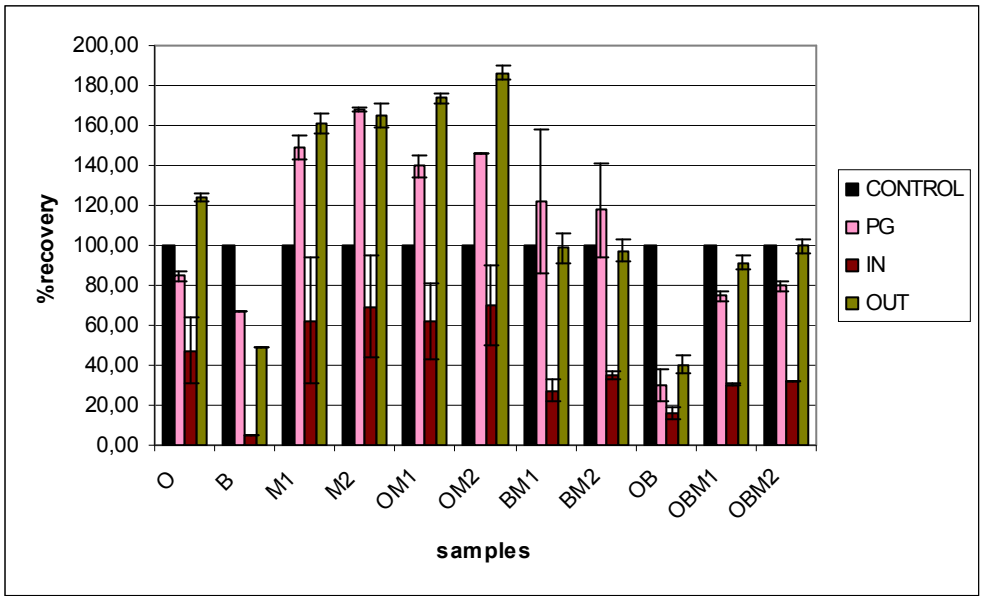


Figure 4. 9 : Recovery of total phenolic content after bioaccessibility assay.

Oat meal was found to have higher recovery values than blueberry. The increase in total phenolic content in intestinal conditions was in agreement with a study on millet grain accessibility conducted by Chandrasekara and Shahidi (2012). The total phenolic content of millet grains were found to be increased significantly after gastrointestinal digestion and having a 2-15% more phenolic content according to 70% acetone exracts of the samples. In the study, OUT sample was found to have approximately 24% more phenolic content according to 70% acetone extracts. The difference can be sourced from different cereal samples used in studies.

Whole milk and skimmed milk had higher total phenolic content recovery in PG, OUT than control samples after bioaccessibility assay and also had a higher total phenolic content recovery in IN sample compared to oat meal and blueberry. This effect might be sourced from antioxidative peptides derived from digestion of milk proteins (Pihlanto, 2006).

4.6.2. Effect of codigestion on bioaccessibility for total phenolic content.

Codigestion with whole and skimmed milk changed the bioaccessibility of phenolics from oat meal and blueberry samples. The effect of whole and skimmed milk on potential bioavailability was investigated by comparing estimated expected values and observed values. In addition, OB sample was analyzed to investigate the difference between observed and expected values. Expected values were calculated by using the seperate phenolic content results of O, B and OB samples after in vitro digestion process. The M1 and M2 samples were also added in calculations due to their positive total phenolic content results after in vitro digestion. Expected values were calculated by multipling the original values and ratios of each ingredient in the mixture and dividing by total. For example, the expected value of BM1 was calculated by multipling the total phenolic content of B and M1 with their ratios in mixture (for B;1 and for M1;8) and then dividing by total ratio (9). The differences between observed values and expected values were analyzed statistically by using one way ANOVA (p <0.05).

When statistical difference was observed, higher observed values implied synergistic, lower observed values than expected values implied inhibition effect of milk. When there was no statistically significant difference between observed and expected values, it was expressed as an additive effect of milk. The expected and observed values and effect of whole, skimmed milk are shown in Table 4.18. The results were shown as mg gallic acid equivalents/ g fresh weight (mg GAE/g fw).

According to results of OB sample, it was possible to say codigestion of blueberry with oat meal resulted in inhibition effect for PG and OUT samples.This finding was in accordance with a previous study reporting the negative effect of dietary fiber on bioaccessibility of polyphenols from fruits and vegetables. For PG samples, observed value was lower than expected values significantly. It might be sourced from limiting effect of dietary fiber in oat sample. Dietary fiber was reported to be responsible for low bioaccessibility of antioxidative substances by limiting their release from food matrices. For OUT samples, the result was similar to PG samples, this finding could also be explained by effect of dietary fiber. Dietary fiber was also thought to catch phenolic compounds and cause to obtain lower bioaccessibility values (Palafox-Carlos *et al*., 2011). However, codigestion had no significant effect on total phenolic content of IN sample, this finding was agreement with the study conducted by

McDoughall *et al*. (2005) investigating the codigestion effect of raspberry with bread.

Table 4.18 : The phenolic content of PG, IN, OUT samples from blends.

Samples		PG (mg GAE/g fw)		IN (mg GAE/g fw)		OUT (mg GAE/g fw)	
OM1	E	0.82±0.03	(Ad)	0.36±0.17	(Ad)	0.99±0.01	(Ad)
	O	0.90±0.04		0.40±0.12		1.11±0.01	
OM2	E	0.85±0.01	(In)	0.37±0.14	(Ad)	0.98±0.01	(Sy)
	O	0.90±0.00		0.43±0.12		1.14±0.02	
BM1	E	1.62±0.03	(Ad)	0.36±0.15	(Ad)	1.43±0.03	(In)
	O	1.27±0.38		0.28±0.06		1.03±0.08	
BM2	E	1.67±0.00	(Ad)	0.38±0.11	(Ad)	1.41±0.03	(In)
	O	1,11±0.22		0.33±0.01		0.91±0.05	
OB	E	2,36±0.02	(In)	0.46±0.14	(Ad)	2.24±0.01	(In)
	O	0.87±0.23		0.45±0.09		1.17±0.13	
OBM1	E	0.82±0.11	(Ad)	0.38±0.14	(Ad)	0.97±0.04	(Ad)
	O	0.91±0.03		0.37±0.00		1.11±0.04	
OBM2	E	0.85±0.09	(Ad)	0.39±0.11	(Ad)	0.96±0.03	(Sy)
	O	0.92±0.03		0.37±0,00		1.15±0.04	

Datas are expressed as mean + SD. Datas examined for a significant difference between O and E values ($p<0,05$), E, expected value;Sy, synergistic effect; Ad, additive effect; In, inhibition.

Whole milk and skimmed milk were generally found to have inhibition effect on the total phenolic content of samples except for the additive effect of skimmed milk on oat sample previously. Contrary to results of milk effect on total phenolic content as discussed before, milk had a varied effect on total phenolic contents of combined samples after bioaccessibility assay. For BM1 and BM2 samples, milk addition was found to have no significant effect on total phenolic content of PG and IN samples after in vitro digestion assay. In other words, milk had no effect on the potential bioavailability of blueberry samples. However, OUT results were significantly lower