ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

PHENOLIC PROFILE, ANTIOXIDANT ACTIVITY AND BIOACCESSIBILITY OF PEELS AND FLESHES OF A LOCAL RED BEET VARIETY NATIVE TO TURKEY

M.Sc. THESIS

Kadriye Nur KASAPOĞLU

Department of Food Engineering

Food Engineering Programme

Thesis Advisor: Prof. Dr. Beraat ÖZÇELİK

JANUARY, 2015

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

PHENOLIC PROFILE, ANTIOXIDANT ACTIVITY AND BIOACCESSIBILITY OF PEELS AND FLESHES OF A LOCAL RED BEET VARIETY NATIVE TO TURKEY

M.Sc. THESIS

Kadriye Nur KASAPOĞLU
(506121516)

Department of Food Engineering

Food Engineering Programme

Thesis Advisor: Prof. Dr. Beraat ÖZÇELİK

JANUARY, 2015

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

TÜRKİYE'YE ÖZGÜ YÖRESEL BİR PANCAR ÇEŞİDİNDE VE ATIKLARINDA FENOLİK MADDE PROFİLİNİN, ANTİOKSİDAN KAPASİTESİNİN VE BİYOYARARLILIĞIN BELİRLENMESİ

YÜKSEK LİSANS TEZİ

Kadriye Nur KASAPOĞLU
(506121516)

Gıda Mühendisliği Anabilim Dalı

Gıda Mühendisliği Programı

Tez Danışmanı: Prof. Dr. Beraat ÖZÇELİK

OCAK, 2015

Kadriye Nur KASAPOĞLU, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID **506121516**, successfully defended the **thesis/dissertation** entitled "**DETERMINATION OF PHENOLIC PROFILE, ANTIOXIDANT ACTIVITY AND BIOAVAILABILITY OF PEELS AND FLESHES OF A LOCAL RED BEET VARIETY NATIVE TO TURKEY**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Beraat ÖZÇELİK** İstanbul Technical University	.....
Jury Members :	**Assoc. Prof. Esra ÇAPANOĞLU GÜVEN** Istanbul Technical University	.....
	Dr. Banu BAYRAM Tübitak	.....

Date of Submission : 15 December 2014
Date of Defense : 23 January 2015

To my Professor and family,

FOREWORD

Firstly, I would like to express my appreciation and special gratitude to my supervisor, Prof. Dr. Beraat ÖZÇELİK for her guidance and support throughout this study. It is my pleasure to be a student of her.

I would also like to thank my collegues Mine GÜLTEKİN ÖZGÜVEN, Nalan DEMİR, Evren DEMİRCAN and Assoc. Prof. Dr. Esra ÇAPANOĞLU GÜVEN for their cooperation and support.

Finally, I would like to thank my family for their continuous encouragement and support throughout my education as well as this study.

December 2014 Kadriye Nur KASAPOĞLU

TABLE OF CONTENTS

Page

ABBREVIATIONS

ANOVA : Analysis of Variance
BVf **:** Beta Vulgaris flesh
BVp **:** Beta Vulgaris peel
BC **:** Betacyanin
BX **:** Betaxanthin
CUPRAC **:** Cupric Reducing Antioxidant Capacity
DPPH **:** 2,2-diphenylpicrylhydrazyl
DW **:** Dry weight
FC **:** Folin-Ciocalteu
FW **:** Fresh weight
GAE **:** Gallic acid equivalent
GI **:** Gastro-intestinal
HPLC **:** High Performance Liquid Chromatography
MS **:** Mass Spectrometry
µg **:** Microgram
ng **:** Nanogram
SD : Standard Deviation
SPSS **:** Statistical Package for the Social Sciences
TAC : Total antioxidant capacity
TBC **:** Total Betalain Content
TPC **:** Total Phenolic Content
TE **:** Trolox equivalent
UFLC **:** Ultra Fast Liquid Chromatography

LIST OF TABLES

Page

LIST OF FIGURES

Page

DETERMINATION OF PHENOLIC PROFILE, ANTIOXIDANT ACTIVITY AND BIOAVAILABILITY OF PEELS AND FLESHES OF A LOCAL RED BEET VARIETY NATIVE TO TURKEY

SUMMARY

Consumption of vegetables is considered as a traditional healthy diet because of the numerous nutrient substances they possessed. The health promoting action of fruits and vegetables has been attributed to the presence of phytochemicals, vitamins and enzymes, as well as other minerals and fibers. These compounds have strong protective effects against major disease risks including cancer and cardiovascular diseases due to their rich potential as antioxidants. Polyphenols are present in fruits and vegetables in great abundance as powerful antioxidants in addition to the other constituents. Plants bearing polyphenols and other phytochemicals with antioxidant potential are gaining attention of researchers gradually.

Beetroot (*Beta vulgaris L.*) contains water-soluble nitrogen-containing pigments called as betalains as well as polyphenolics compounds. Betalains are known to have antioxidant, antimicrobial, antiviral, antiproliferative, anti-inflammatory, hepatoprotective, and anti-cancer activities. Due to these excessive health-promoting effects, new betalain-containing food products are of interest. A local red beet variety native to Turkey has been investigated for the first time including fleshes (BVf) and peels (BVp). The experiments were carried out to three different biological replicates which were harvested at different times from various farms. The results of all assays indicated statistically significant differences between the batches and between peel and flesh samples. Peel samples showed higher values than flesh samples according to all assays performed.

Phenolic profile was analyzed using UFLC-MS/MS. Similar chromatogram patterns of phenolic acids were observed for peel and flesh samples; contents differed depending on batches. As a result, the presence of chlorogenic acid, ferulic acid, syringic acid, vanillic acid and 4(p-)hydroxybenzoic acid were detected in both types of extract (BVp and BVf) at different concentrations. However, caffeic acid and rutin were present only in trace amounts. Notably, chlorogenic and ferulic acid was the major phenolic constituents for BVp samples whereas ferulic acid was the only major phenolic in BVf samples in contrast with red beetroot, which contains 4-hydroxybenzoic acid as the major phenolic acid. The phenolic acid composition was seen different from the red beet and sugar beet. The decreasing order of individual phenolic acids in peels was as follows: ferulic acid, chlorogenic acid, syringic acid,

4(p-)hydroxybenzoic acid, vanilic acid. On the other hand, the decreasing order of individual phenolic acids in fleshes was as Ferulic acid, syringic acid, vanilic acid, 4(p-)hydroxybenzoic acid, chlorogenic acid.

In regards to the total antioxidant capacity methods, CUPRAC assay gave higher responses than the DPPH assay in accordance with the literature, although the antioxidant capacity of this beetroot variety was not as high as red beetroot. Total phenolic content was in a similar range with the reported values for red beetroot. DPPH Free Radical Scavenging Capacity and CUPRAC Cupric Reducing Antioxidant Capacity assays were performed to determine the antioxidant capacity and the total phenolic content was determined via Folin-Ciocalteu method.

The bioaccessibility of phenolics from both flesh and peel parts was assessed by in vitro gastro-intestinal digestion procedure that simulates the physiochemical and biochemical changes that occur in the gastrointestinal tract. Since in vivo studies on humans and animals are not preferred due to the limitations such as complexity, expensiveness and ethical reasons, in vitro digestion assays provide rapid and simple methods to evaluate phytochemical stability in food matrices. Results from in vitro digestion demonstrated that differences in bioaccessibility of polyphenolics between the batches were significant and the reduction of TPC in peel samples is greater than in fleshes. In other words, the bioaccessibility of phenolics in fleshes is greater than the bioaccessibility of phenolics in fleshes. Additionally, phenolic acids were found to be stable in gastric digestion due to acidic conditions. However, their recoveries become lowered in IN fractions representing the compound passed into serum. The UFLC-MS/MS detection was done on phenolic acids from PG, IN and OUT fractions of GI digestion. Notably, no peak could be detected in IN, OUT and PG fractions for the 4(p-)hydroxybenzoic acid. Furthermore, the total betalain content was quantified spectrophotometrically and expressed as the sum of red-violet (betacyanin -BC) and yellow-orange (betaxanthin -BX) pigments. BX and BC of peel samples were found as 1.6 ± 0.01 mg g-1 indicaxanthin equivalent and 0.9 ± 0.01 mg g-1 betanin equivalent whereas 0.3 ± 0.05 mg g-1 indicaxanthin equivalent and 0.9 ± 0.01 mg g-1 betanin equivalent for flesh samples, respectively.Total betalain content of this beetroot variety was found as 2.5 mg/ g for peels and 1.2 mg/ g for fleshes, lower than the red beetroot.

TÜRKİYE'YE ÖZGÜ YÖRESEL BİR PANCAR ÇEŞİDİNDE VE ATIKLARINDA FENOLİK MADDE PROFİLİNİN, ANTİOKSİDAN KAPASİTESİNİN VE BİYOYARARLILIĞIN BELİRLENMESİ

ÖZET

Sebze tüketimi, sebzelerin sahip olduğu sayısız besleyici içerik sayesinde sağlıklı ve geleneksel bir beslenme biçimidir. Sebzelerin ve meyvelerin bu sağlığa yararlı etkisi fitokimyasallar, vitaminler ve enzimlerin yanı sıra mineraller ve life de dayanır. Bu bileşimler kanser ve kardiyovasküler hastalık risklerine karşı içerdikleri zengin antioksidanlar sayesinde yüksek koruma sağlarlar. Polifenoller meyve ve sebzenin içinde diğer bileşenlere ek olarak antioksidan aktivite sağlarlar. Antioksidan potansiyel gösteren polifenolleri taşıyan bitkiler giderek araştırmacıların daha çok dikkatini çekmektedir. Kırmızı pancar (*Beta vulgaris L.*) fenoliklerin yanı sıra suda-çözünür azot ihtiva eden antioksidan, anti-mikrobiyal, anti-viral, anti-prolifetarif, anti-iltihabik, anti-kanser ve karaciğeri koruyucu etkilere sahip olduğu bilinen betalainler olarak adlandırılan pigmentleri içermektedir. Tüm bu sağlığa olumlu etkileri dolayısıyla, yeni betalain içeren gıda ürünlerine karşı artan ilgi söz konusudur.

Türkiye'ye özgü bir yöresel kırmızı pancar çeşidinde iç kısım (BVf) ve kabuklar (BVp) bu çalışma kapsamında ilk kez araştırılmıştır. Bilgimize göre, bu varyeteye ait herhangi bir çalışma literatürde bulunmamaktadır. Deneyler, farklı zamanlarda ayrı yerlerden hasat edilen numunelerle üç farklı biyolojik tekrar olarak yapılmıştır. Tekrar yığınların sonuçları arasında ve kabuk ile iç kısım arasında tüm deneylere göre istatistiksel olarak önemli farklar izlenmiştir. Kabuk kısımları bütün analizlerde (toplam fenolik, toplam betalain, ve antioksidan kapasite) iç kısımlardan daha yüksek değerler vermiştir.

Fenolik profil UFLC-MS/MS kullanılarak analiz edilmiştir. Kabuk ve iç kısımlarda benzer fenolik asit kromatogramları gözlenmiş olup, fenolik asit miktarları yığınlara göre farklılık göstermiştir. Sonuç olarak, sinnamik asit, klorojenik asit, vanilik asit, 4-hidroksi-benzoik asit, trans-ferulik asit ve kafeik asit hem iç hem kabuk kısımlarında farklı konstrasyonlarda izlenmiştir. Bununla birlikte, kafeik asit ve rutin sadece iz miktarda gözlenmiştir. Özellikle, klorojenik ve ferulik asit kabuk kısımları için en yüksek miktardaki fenolik bileşenlerdir. Kırmızı pancarda ise en yüksek miktardaki fenolik asit 4(*p*-)hidroksi-benzoik asit olarak bildirilmiştir. Bu varyetenin

fenolik asit kompozisyonunun kırmızı pancar ve şeker pancarından farklı olduğu bulunmuştur. Kabuk için fenolik asit miktarları azalan sırayla: ferulik asit, klorojenik asit, şirincik asit, 4(p-)hidroksibenzoik asit, vanilik asit olarak izlenirken, iç kısım içinse: ferulik asit, şirincik asit, , vanilik asit, 4(p-)hidroksibenzoik asit, ve klorojenik asit olarak izlenmiştir. Gallik asit, elajik asit, prokateşuik asit, sinapik asit, trans-sinamik asit, ve *p*-kumarik asit ise tespit edilememiştir.

Toplam antioksidan kapasite analizlerinde, literatürle benzer şeklide DPPH yöntemi, CUPRAC yönteminden daha düşük sonuçlar vermiştir. Ancak, antioksidan kapasitenin kırmızı pancarda olduğu kadar yüksek olmadığı görülmüştür. Toplam fenolik madde miktarı kabuk için 8.50 mg GAE/ g DW iken iç kısım için 5.45 mg GAE/ g DW olarak bulunmuştur. Bu değerler yüksek olmamakla birlikte, kırmızı pancar için literatürde bildirilen aralığa girmektedir. Spektrofotometrik yöntemler olan DPPH ve CUPRAC metodları toplam antioksidan kapasitesini belirlemek için kullanılmıştır. Literatürde antioksidan kapasite tayini için pancar numunelerinde en çok DPPH yöntemi kullanılmıştır. CUPRAC metodu ise asetat tampon çözelti sayesinde fizyolojik pH ortamında analiz sağladığı için sebze-meyve numunelerinde tercih edilmektedir. Toplam fenolik madde içeriği ise Folin-Ciocalteu metodu ile belirlenmiştir. Folin-Ciocalteu metodu, numunede bulunan fenolikleri gallik asit eşdeğeri cinsinden toplam miktar olarak tayin eder.

Pancarın hem iç kısım hem de kabuklarında bulunan fenoliklerin biyoyararlılığı *in vitro* sindirim metodu kullanılarak ağız, mide ve ince bağırsak sindirimi fizyokimyasal ve biyokimyasal anatomi simüle edilerek incelenmiştir. Insan ve hayvan gibi canlı organizmada yapılan araştırmalar karmaşıklık, pahalılık ve etik sebeplerden dolayı tercih edilmediğinden, *in vitro* metodu deney ortamında sindirimi çözümleyip hızlı ve basit yöntem sunarak bitki matrislerinde değerlendirmede olanağı sağlar. *In vitro* sindirim metodu sonuçlarına göre, yığınların biyoyararlılığı arasındaki farklar önemlidir. Ayrıca, kabuk numunelerde fenolik madde miktarındaki azalma iç kısımlardan fazladır. Yani, iç kısımların biyoyararlılığı kabuk kısımlarından yüksektir. Fenolik asitler mide sindiriminde asidik ortam şartlarından dolayı stabildirler. Ancak, IN fraksiyonlarında, yani bağırsak içinde emilmek üzere bulunan fenolik miktarlarında azalma meydana gelmekle beraber, incelenen numunede bulunan fenolikler kullanılabilir orandadırlar.

In vitro sindirim sonrası UFLC-MS/MS analizinde 4(*p*-)hidroksibenzoik asit IN, PG ve OUT fraksiyonlarında izlenmemiştir. Toplam betalain içeriği spektrofotometrik yöntem ile ölçülerek kırmızı-mor (betacyanin -BC) ve sarı-turuncu (betaxanthin -BX) pigmentlerin toplamı olarak ifade edilmiştir. Betaxantin ve betasiyanin içeriği kabuk için sırasıyla 1.6 ± 0.01 mg g^{-1} indicaxantin eşdeğeri ve 0.9 ± 0.01 mg g^{-1} betanin eşdeğeri olarak bulunurken, iç kısım için 0.3 ± 0.05 mg g^{-1} indicaxantin ve 0.9 ± 0.01 mg g^{-1} betanin olarak bulunmuştur. Toplam betalain içeriği kabuk için 2.5 mg/ g ve iç kısım için 1.2 mg/ g olarak bulunmuştur. Bu sonuç, kırmızı pancara göre oldukça düşük olmakla beraber, literatürde araştırılan diğer betalain içeren bitki,

sebze ve meyvelere kıyasla değerlidir. Güncel olarak, sağlık bakımından pek çok fayda ihtiva eden betalain pigmenti eldesiyle ilgili pek çok çalışma yapılarak yeni kaynaklar araştırılmaktadır. Bu bağlamda, yalnızca toplam betalain içeriği değil, BX/BC pigmentlerinin oranı da farklı renk skalası elde etme bakımından önem teşkil etmektedir.

1. INTRODUCTION

The work in this M.Sc. project has focused on a local red beetroot variety native to Turkey. Beetroot (*Beta vulgaris subsp. vulgaris*) originates from the Mediterranean Sea region, is an important vegetable which has been cultivated for many hundreds of years in temperate climates (Kujala et al., 2001; Janiszewska, 2014). Epidemiological data showed that the consumption of a plant-based diet is essential for human health providing various phytochemicals. The suggested advantageous health effects include maintenance of health and protection from diseases such as cancer and coronary heart disease due to antioxidant activities of plant polyphenolics. Beetroots are pigmented foods, they possess characteristic betalain pigments in addition to plant phenolics (Tesoriere et al., 2008).

Betalain pigments in beetroot exhibit excessive health promoting activities such as antioxidant, antimicrobial, antiinflammatory, antiatherosclerotic, antitumour and hepatoprotective effect (Janiszewska, 2014). Red beetroot concentrate is universally permitted as a food ingredient as beetroot red (Kujala et al., 2000). Vinson et al. (1998) ranked beets among the ten most potent vegetables with respect to antioxidant activity. Therefore, several research studies have been performed on red beetroot (Kujala et al., 2002; Gandia-Herrero et al., 2010; Georgiev et al., 2010; Canadonovic et al., 2011; Nemzer et al., 2011; Ravichandran et al., 2013; Ben Haj Koubaier et al., 2014) however, other varieties of beetroots remain unknown in terms of polyphenolic and betalain content to our knowledge. Stintzig, Schieber and Carle (2000) identified betalains from yellow beet. Being widely used in food industry as food ingredients, especially colorants, beetroots have a large potential to expand their utilization by local varieties.

In human body, bioactive compounds such as polyphenols obtained from the diet are extracted following a gastrointestinal (GI) digestion. The compounds must be biologically accessible to show the possible health benefits. Bioaccessibility specifically refers to the amount of antioxidants which are potentially presented to the intestinal

brush border for absorption (Wootton-Beard and Ryan, 2011). The *in vitro* digestion model gives an indication to the bioaccessibility since it is well correlated with the results obtained from human studies and animal models (Bouayed *et al.*, 2011). Based on our current knowledge, no previous study is available that determines the phenolic profile, antioxidant activity and *in vitro* bioaccessibility of this local beetroot variety using the simulation of *in vitro* gastrointestinal (GI) digestion.

1.1 Purpose of Thesis

The objective of this study is to explore an underutilized local red beet variety in terms of antioxidant capacity related to the valuable betalain pigments and phenolic acids as well as the bioavailability proposing a multifunctional food additive.

1.2 Literature Review

1.2.1 Beetroot

A root is the underground storage system of a plant. Plant root system functions primarily as water and nutrient absorption from the surrounding media. A root consists of an inner core called the xylem surrounded by the phloem, and an outer layer of epidermis (Rubatzky et al. 1999). In beetroot (*Beta vulgaris L.*), concentric circles of xylem and phloem surrounded by parenchyma cells are produced resembling the growth rings of a tree (Raven et al. 2005). A schematic diagram of the root morphology is seen in Figure 1.1.

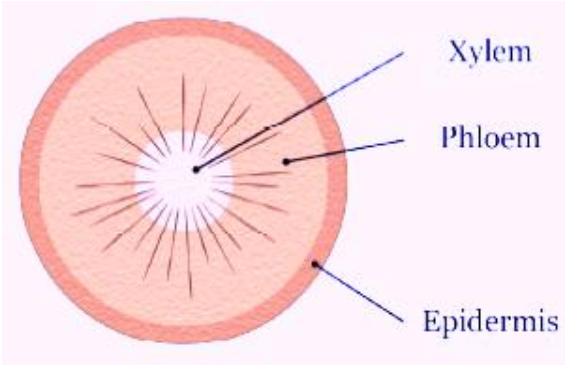


Figure 1.1 : Schematic drawing of the morphology of a taproot. (Bach, 2012)

Beetroot is ranked as one of the 10 most potent vegetables, because of its total phenolic content of 50–60 μmol/g dry matter (Vulic et al., 2012). The edible roots contain from12 to 20% dry matter, including 4–12% sugar, 1.5% protein, 0.1% fat, 0.8% fibre, minerals such as sodium, potassium, phosphorus, calcium, and iron, as well as small amounts of vitamins. As other plant products, beetroots contain bioactive polyphenolic phytochemicals that are beneficial for health. Due to antioxidant activities of polyphenolics, maintenance of health and protection from diseases such as cancer and coronary heart disease were reported in the literature. Beetroots are pigmented vegetables, they possess characteristic pigments called as betalains in addition to the plant phenolics (Tesoriere et al., 2008).

1.2.2 Betalains

Beetroot is a great source of valuable water-soluble nitrogenous pigments, called betalains. The two groups of betalain pigments are red-violet betacyanins (Latin Beta, beet and Greek kyanos, blue color) and yellow-orange betaxanthins (Latin Beta, beet and Greek xanthos, yellow color) which are betalamic acid derivatives. Their content in the roots depends on the degree of maturity, variety and climatic conditions (Cai, et al., 2003; Gokhale and Lele, 2011; Krajka-Kuzniak et al., 2012; Ravichandran et al., 2013; Serris and Biliaderis, 2001). More than 50 natural betalains have been described since the early 1960s. Betalamic acid [4-(2-oxoethylidene)-1,2,3,4-tetrahydropyridine-2,6-dicarboxylic acid] is a naturally occurring compound that is found condensed with amino acids, amines, cyclo-DOPA (cyclic 3,4-dihydroxyphenylalanine), and cyclo-DOPA derivatives to form the betalains (Gandia-Herrero et al., 2012). Chemical structures of betalamic acid (the chromophore of all betalains), betacyanins, and betaxnathins are shown in the Figure 1.2.

The most important factor affecting pigment synthesis in beet tissues has been reported to be the temperature during the growing season in addition to environmental factors such as soil fertility, soil moisture, irrigation and harvest dates. A significant root-to-root variability in pigment content of several cultivars was reported (Kujala, Vienola, Klika, Loponen and Pihlaja, 2002).

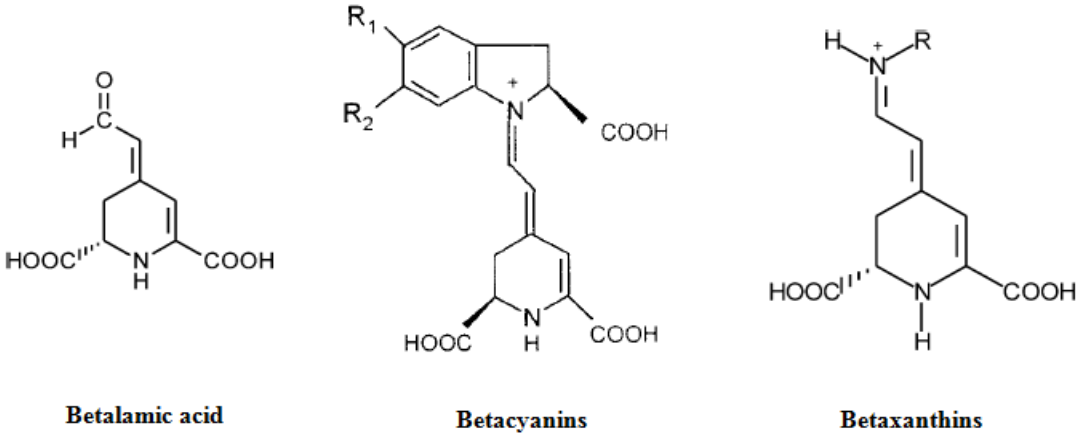


Figure 1.2 : Chemical structures of betalamic acid, betacyanins and betaxanthins (Strack et al., 2003).

The total betacyanin and betaxanthins content of red beetroots differs within the ranges of 47-58 mg (100 g d.m.)-1 for betaxanthins and 525-875 mg (100 g d.m.)-1 for betacyanin, depending on the cultivar. Like the other pigments, betalains are sensitive to external factors, mainly temperature and oxygen, and their stability depends on pH (from 3 to 7) (Janiszewska and Wlodarczyk, 2013.) Different redness shades of the root flesh are determined by the ratio of betacyanins to betaxanthins, and their relative content depends on root size, variety, and weather conditions during cultivation and fertilization (Felczyński and Elkner 2008). Nowadays, the use of natural pigments for food coloring receives more and more attention as the synthetic dyes become critically assessed by the consumers. In food industry, anthocyanins are commonly used natural pigments covering the red-purple color range, however betalains are more stable to pH and temperature. Betalains exhibit broad pH (3-7) stability which are well suited for low-acid foods where coloring with anthocyanins usally not possible. (Stintzing & Carle, 2004) For the yellow-orange color, carotenoids are the natural pigments used as dyes but due to poor solubility in water, betaxanthin could be used in application as yellow-orange food colorants in situations (Azeredo, 2009).

Betalains are characteristic for the plant order Caryophllales and a mutual exclusion of betalains and anthocyanins is present in the nature (Kuajala et al., 2001). The Caryophyllales-specific occurrence of betalains is a noticeable example of the

chemotaxonomic relevance of plant secondary products (Strack et al., 2003). The structure of betalains is completely different from that of the anthocyanins and flavonoids present in most other plants, with the molecular formula $D_{24}H_{26}O_{13}N_2$ containing three carboxyl groups (Gasztonyi et al., 2001). Of the various natural sources of betalains, red beetroot concentrate is universally permitted as a food ingredient, termed as beetroot red (Kujala et al.,2000). Betanin, a principle betacyanin pigment, -containing beet root extracts are used to give a pink or violet color to foods and beverages as the additive 73.40 in the 21 CFR section of the Food and Drug Administration (FDA) in the United States and under the E-162 code in the European Union. Besides, betaxanthins have also been proposed as new colorants (Gandia-Herrero et al., 2010).

Having several health benefits, the presence of betalains is restricted to a few edible vegetables and fruits such as red beet (Beta vulgaris subsp. vulgaris), cactus pear (Opuntia ficus indica), ulluco (Ullucus tuberosus), chard (Beta vulgaris subsp. cicla) and pitaya, they receive a growing interest in the use of natural pigments for food colorants as food additives (Tesoriere et al., 2004). The desirable biological activities that betalains possess include antioxidant, antimicrobial, antiinflammatory, hepatoprotective, antiatherosclerotic and antitumour properties (Canadanovic-Brunet et al., 1998; Kugler et al., 2004; Svenson et al., 2008; Stintzing et al., 2002; Tesoriere et al., 2004).

Figure 1.3 : Different beetroot varieties.

In the literature, red beetroot is the most studied betalain source by plenty of researchers (Canadanovic-Brunet et al., 1998; Czapski et al., 2009; Gasztonyi et al., 2001; Georgiev et al., 2010; Kujala et al., 2000; Kujala et al., 2001; Pedreno & Escribano, 2000; Ravichandran et al., 2013; Vulic et al., 2012) regarding the antioxidant capacity of betalains as well as phenolics. On the other hand, the bioaccessibility of red beet betalains investigated by Tesoriere et al. (2004; 2008) and Vulic et al. (2014). However, scarce studies present about the varieties with red-violet and white alternating rings that is subject of this research. Regarding the morphology, the shape of the investigated beetroot variety ranged between oblong and flat-spherical. The peel differs from the pulp with a darker red color whereas the flesh is rich with stripes of red-violet and white color.

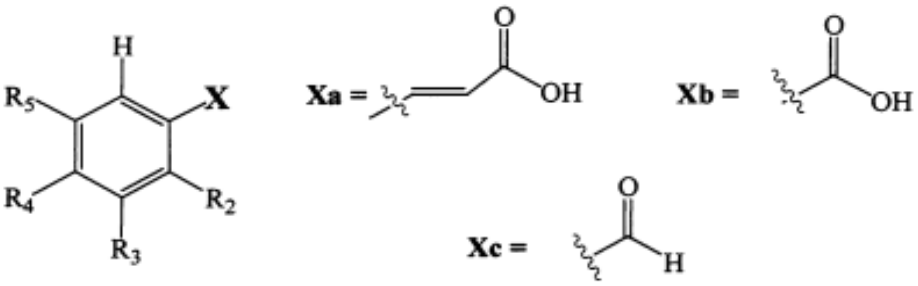
1.2.3 Phenolics

Along with the betalain pigments, phenolic acids and phenolic acid conjugates have also been reported in different beet materials (Kujala et al., 2000; Ng et al., 1998; Wende et al., 1999). Phenolic acids are also powerful antioxidants and have been reported to demonstrate antibacterial, antiviral, anticarcinogenic, anti-inflammatory, anti-aging and vasodilatory actions (Duthie, Duthie, & Kyle, 2000; Velioglu, Mazza, Gao, & Oomah, 1998), therefore have attracted interest in lately. There are more than eight thousand different polyphenolic compounds identified to date. They contain at least one aromatic ring with one or more attached –OH groups, in addition to other substituents, and can be divided into 15 major structural classes (Apak et al., 2007). Although the basic skeleton remains the same, the numbers and positions of the hydroxyl groups on the aromatic ring create the variety. Phenolic acids can mainly be divided into two classes: derivatives of benzoic acid and derivatives of cinnamic acid (Robbins, 2003). The structures of the prominent naturally occurring phenolic acids are shown in the Table 1.1.

Phenolic quantity and composition of vegetables including beetroot are widely studied (Kähkönen et al., 1999; Vinson, Hao, Su, & Zubik, 1998). Plant phenolics have received more attention in recent years since they are free radical scavengers and prevent active oxygen-induced and free radical mediated oxidation of biological molecules (Pedreno &

Escribano, 2000). In general, antioxidant capacity of these compounds has been attributed to their electron-donating ability. Structure–activity relationship studies have demonstrated that structure and substitution pattern of hydroxyl groups are essential for effective free-radical scavenging capacity (Morales-Soto et al., 2014).

Table 1.1: Structures of the prominent naturally occurring phenolic acids (Robbins, 2003).



R_2	R_3	R_4	R_5	X	code	common name
H	H	H	H	a	**1**	cinnamic acid
–OH	H	H	H	a	**2**	*o*–coumaric acid
H	H	–OH	H	a	**3**	*p*–coumaric acid
H	–OH	H	H	a	**4**	*m*–coumaric acid
H	$–OCH_3$	–OH	H	a	**5**	ferulic acid
H	$–OCH_3$	–OH	$–OCH_3$	a	**6**	sinapic acid
H	–OH	–OH	H	a	**7**	caffeic acid
H	H	H	H	b	**8**	benzoic acid
–OH	H	H	H	b	**9**	salicylic acid
H	H	–OH	H	b	**10**	*p*–hydroxybenzoic acid
H	$–OCH_3$	–OH	H	b	**11**	vanillic acid
H	$–OCH_3$	–OH	$–OCH_3$	b	**12**	syringic acid
H	–OH	–OH	H	b	**13**	protocatechuic acid
–OH	H	H	–OH	b	**14**	gentisic acid
–OH	–OH	–OH	–OH	b	**15**	gallic acid
H	$–OCH_3$	$–OCH_3$	H	b	**16**	veratric acid
H	$–OCH_3$	–OH	$–OCH_3$	c	**17**	syringealdehyde
H	$–OCH_3$	–OH	H	c	**18**	vanillin

Phenolic substances can vary in different varieties and cultivars in plants. Additionally, contents of phenolic substances are influenced by a large number of external factors such as agrotechnical processes, climatic conditions and ripeness during harvest, postharvest manipulations, and time of consummation (Morales-Soto et al., 2014). Kahkönen et al. (1999) indicated that the total phenolic contents of vegetables were very low compared to fruits. Vinson, Hap, Su, and Zubik (1998) found that beets had the highest total phenolic content, followed by red onion,

broccoli and kidney beans. On the other hand, Velioglu et al. (1998) reported that red onion had a higher total phenolic content than other plant materials. Sreeramulu and Ragnutah (2010) compared some root and tuber vegetables that are commonly consumed in India in terms of total phenolic content and antioxidant capacity. Red beetroot (*Beta vulgaris*) gave the highest results of TPC followed by tapioca (*Manihot esculenta*), colacasia (*Colacasia antiquorum*), spring onions (*Allium cepa*), white radish (*Raphanus sativus*), onions (*Allium cepa*), yam (*Typhonium trilobatum*), sweet potato (*Ipomoes batatas*), potato (*Solanum tuberosum*), carrot (*Daucus carota*).

Red beetroot contains phenolic acids including *p*-coumaric, protocatechuic, ferulic, vanillic, p-hydroxybenzoic and syringic acids (Kujala et al., 2000; Vulic et al., 2012). 4-hydroxy benzoic acid was reported as the major phenolic constituent in red beet followed by cinnamic acid, vanillic, chlorogenic, trans ferulic acid, caffeic acid, and epicatechin. Coumaric and anis acids were founded only in trace amounts (Georgiev et al., 2010; Kujala et al., 2000; Ravichandran et al., 2012). On the other hand, *p*-coumaric acid and ferulic acid were detected in the peels of red beetroot (Kujala, Loponen and Pihlaja, 2001). Obata et al. (1963) determined the phenolic acids in sugar beet molasses. They reported catechol, *p*-hydroxybenzoic acid, 2-hydroxydihydrocinnamic acid, salicylic acid, syringic acid, vanillin and vanillic acid in molasses.

In regards to the total phenolic contents, Folin-Ciocalteu method performed and results were expressed as gallic acid equivalent in the literature. Kahkönen et al. (1999) determined the TPC of red beet peels and sugar beet peels as 4.3 mg GAE per g DW and 4.2 mg GAE per g DW, respectively. Furthermore, Kujala et al. (2000) studied both peels and fleshes of red beetroot and higher phenolic content reported for the peels than fleshes, 15.5 mg GAE per g DW and 4.2 mg GAE per g DW, respectively. For the flesh part, Ben Haj Koubaier et al. (2013) founded the TPC of red beetroot as 14.6 mg GAE per g DW whereas Bavec et al. (2010) reported TPC values between 3.16 - 4.94 mg GAE per g DW in red beetroot. Bavec et al. (2010) studied the effect of different farming systems on flesh of red beet from conventional, integrated, organic, biodynamic, and control farming systems. They reported statistically significant differences in total phenolic content with respect to

the different farming systems. For sugar beet pulp, Mohdaly et al. (2010) determined the phenolic content as 1.79 mg GAE per g DW.

1.2.4 Antioxidant Capacity

Antioxidants are compounds that stop or delay the oxidation processes providing protection against certain oxidative stress-related disorders in human organism as well as several degenerative diseases such as After several studies on the importance of antioxidants in biological systems by counteracting of oxidative stress that causes several human diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders (Karadag, Ozcelik and Saner, 2009). It is known that phenolic plants protect against active oxygen-induced and free radical mediated oxidation of biological molecules and consequently prevents diseases such as cancer and cardiovascular diseases (Georgiev et al., 2010; Janiszewska, 2014; Tesoriere et al., 2014; Vulic et al., 2012). Hence, a great interest present in quantification of antioxidants and determination of antioxidant capacities of a number of specific food compounds.

Many methods have been developed and tested in the literature, still strengths and limitations of these methods have been discussed. No single assay accurately reflects the mechanism of action of all radical sources or all antioxidants in a complex system (Prior et al. 2005). On the basis of the inactivation mechanism involved, antioxidant capacity methods have been generally divided into two classes: (1) hydrogen atom transfer (HAT) reaction and (2) electron transfer (ET) reaction-based methods. Analysis conditions, substrate, and antioxidant concentration should simulate real food or biological systems as much as possible when selecting the antioxidant capacity method. Therefore, the total antioxidant capacity value should include methods applicable to both lipophilic and hydrophilic antioxidants, with regards the similarity and differences of both hydrogen atom transfer and electron transfer mechanism (Karadag, Ozcelik and Saner, 2009).

Research suggest that TPC is an important contributor to the total antioxidant capacity of fruits and vegetables (Kevers et al., 2007; Sreeramulu and Ragnutah,

2010). However, other studies present suggesting lack of correlation between TPC and TAC (Kahkönen et al., 1999; Ismail, Marjan, & Foong, 2004) could be due to the different AOC parameters determined in those studies and/or different responses of phenolic compounds in different AOA assay systems (Kahkonen et al., 1999). There is a wide degree of variation between different phenolic compounds in their effectiveness as antioxidant (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999). Additionally, each type of plant tissue had a different antioxidant activity, contributed by different antioxidant components, such as vitamin C, α-tocopherol, β-carotene or phenolic compounds and total phenolic content estimated by the Folin–Ciocalteu reagent may overestimate TPC (Kaur and Kapoor, 2002). In beetroot, the antioxidant capacity is related to both its betalain pigments and its polyphenol content.

In the literature, broad ranges in antioxidant activities have been reported previously for the same plant material (Cao et al., 1996; Gazzani et al., 1998; Kaur and Kapoor, 2002; Morales-Soto et al., 2014). This could be due to factors like agronomic, genomic and post harvesting conditions which may influence the chemical composition of plant foods (Imeh & Khokhar, 2002; Kahkönen et al., 1999). As the presence of different antioxidant compounds in fruits and vegetables possess difficulties to measure each antioxidant component separately, several methods are used to calculate the total antioxidant capacity of biological samples as well as different extraction mediums to provide the maximum extraction of the available antioxidants from the samples (Gazzani et al., 1998; Velioglu et al., 1998; Vinson et al., 1998; Kahkonen et al., 1999; Kaur and Kapoor, 2002; Morales-Soto et al., 2014).

In the literature, DPPH (2,2-diphenyl-1-picrylhydrazyl) free–radical scavenging capacity assay was used for red beetroot at most (Georgiev et al., 2010; Sreeramulu and Raghunath, 2010; Ravichandran et al., 2012). Sreeramulu and Ragnutah (2010) studied some root and tuber vegetables that are commonly consumed in India using DPPH assay and FRAP (Ferric Reducing Antioxidant Capacity) assays. They concluded that red beetroot (Beta vulgaris) gave the highest results than the other studied samples. Vulic et al. (2012) determined red beetroot pomace, processing by-product from food industry in terms of the phenolic content and betalain content as well as antioxidant capacity in varieties of Cardeal-F1, Egyptian, Bicor, Kestrel

beetroot pomace extracts. Beetroot pomace contained parts of flesh, crown, peel and pulp. . In another study, Vulic et al. (2014) examined the varieties of Detroit, Cardeal-F1, Egyptian, Bicor and Kestrel beetroot pomace extracts using DPPH assay. In accordance with the previous study, they observed that a significant difference exist between the beetroot varieties in terms of antioxidant capacity, phenolic content and betalain content.

Zhou and Yu (2006) compared the total phenolic contents and antioxidant properties of 38 commonly consumed vegetables grown in Colorado including kale, rhubarb, spinach, broccoli, green bean, carrot, tomato, and potato. Besides the antioxidant activity of vegetables determined by different antioxidant assays could give different results, the authors concluded that some of these vegetables that were produced in Colorado may have higher antioxidants than that grown at other locations. However, as far as we know, there is no report on the distinct beetroot varieties that are grown in different geographical locations with regards to the antioxidant properties and polyphenolic properties.

It has been known that both the extraction method and solvent were noticeably affect the content of individual compounds in vegetables (Sulaiman et al., 2011) including beetroots (Castellanos-Santiago and Yahia, 2008; Kujala et al., 2001). For instance, Gorinstein et al. (2009) investigated the antioxidant value and antiproliferative activity of some raw vegetables which were harvested in the same year and in the same geographical and climatic conditions. They observed the effect of acidification in extraction solvent as higher TPC and TAC recoveries.

1.2.5 Bioaccessibility

In human body, bioactive compounds obtained from the diet are extracted following a gastrointestinal (GI) digestion. The compounds must be biologically accessible to show the possible health benefits. Bioaccessibility specifically refers to the amount of antioxidants which are potentially presented to the intestinal brush border for absorption (Wootton-Beard and Ryan, 2011). In recent years, bioaccessibility studies have given evidence of the absorption of any phytochemicals and have shown

that uptake varies widely, depending on the type of compound and/or food matrix (Manach, Williamson, Morand, Scalbert, & Remesy, 2005).

In vivo studies on humans and animals are not preferred due to the limitations of complexity, expensiveness and ethical reasons (Guven et al., 2010). However, the in vitro digestion model gives an indication to the bioaccessibility since it is well correlated with the results obtained from human studies and animal models and a rapid and simple method to evaluate phytochemical stability in (Bouayed et al., 2011). The in vitro digestion model simulates stomach digestion using pepsin and mimickes intestinal digestion using pancreatin and bile salts.

Bouayed et al. (2012) evaluated bioaccessible and dialysable apple polyphenols available for potential uptake by intestinal epithelial cells using in vitro gastrointestinal (GI) digestion method. They observed that polyphenolic profiles in the gastric medium were similar to those natively occurring in apples; however, bioaccessible polyphenols were at lower concentrations than those in the apples. Moreover, the polyphenolic profile was altered during intestinal digestion, with a considerable decrease of total polyphenols.

To our knowledge, no study exist determining the phenolic content and composition, antioxidant activity and in vitro bioaccessibility of this local beetroot variety using the in vitro gastrointestinal (GI) digestion. Wootton-Beard and Ryan (2011) examined the total antioxidant capacity and total polyphenol content of 23 commercially available vegetable juices including beetroot juice, vegetable juice, apple juice, tomato juice and carrot juice following in vitro digestion. All vegetable juices observed to be a significant source of antioxidants, regardless of price, storage or processing conditions. However, a wide variation in TAC and TPC between different types of juice was reported. As a result, beetroot juice displayed by far the highest antioxidant capacity across all of the assays conducted among other vegetable juices, and has an antioxidant capacity similar to, or greater than that of pomegranate or cranberry juice. They observed that TAC and TPC of the shot increased significantly following the in vitro digestion procedure despite a decrease between the gastric and duodenal phases. Notably, they did not elucidate the change in the phenolic composition after the *in vitro* GI digestion.

1.3 Hypothesis

The hypothesis is that an understanding of the chemical and nutritional value of a local red beet variety in order to contribute new knowledge on a new food crop to increase preference and utilization in the food industry as a multifunctional food additive.

The main aim is to provide a chemical approach to understand the chemical alteration depending on antioxidant activity as an effect of beetroot variety, divided into the following sub-aims:

- To quantify the phenolics and betalain content in order to elucidate the total antioxidant capacity and to determine the phenolics profile.
- To examine the bioaccessibility based on the stability of polyphenolics and antioxidant capacity through *in vitro* digestion.

In order to achieve these specific aims, several analytical methods were applied: analysis of total betalain content using spectrophotometry, analysis of individual phenolic acids by high performance liquid chromatography UFLC-MS/MS system which is equipped with an ultra fast liquid chromatography (Prominence Liquid Chromatography LC-20AD, Shimadzu), analysis of total phenolics by the Folin-Ciocalteu (FC) method, analyses of total antioxidant capacity by both Free Radical Scavenging Capacity (DPPH) and Cupric Reducing Antioxidant Capacity (CUPRAC) assays, analysis of bioavailability by in vitro gastrointestinal digestion model.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Folin-Ciocalteu reagent, gallic acid, 2,2 diphenyl-1-picrylhydrazyl, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich Chemical Co (Steinheim, Germany). Other chemicals and reagents were of analytical or high-performance liquid chromatography grade. For the simulation of in vitro gastrointestinal digestion system, dialysis bags (Dialysis tubing cellulose membrane, 1.3 in.) were purchased from Sigma-Aldrich Chemical Co (Steinheim, Germany). All chemicals and reagents used for the analyses were of analytical or high-performance liquid chromatography (HPLC) grade and obtained from Sigma-Aldrich Chemie GmbH & Co. KG (Steinheim, Germany), unless otherwise specified.

2.2 Plant Material

The red beet plants (Beta vulgaris L.) were harvested as three different biological replicates in 2nd, 3rd and 4th weeks of November 2014 from different local farmers in Siirt, Turkey.

Figure 2.1 : Red beetroot samples used.

The beetroots were carefully washed and hand-peeled, and both the peels (BVp) and fleshes (BVf) were cut into small pieces and powdered in liquid nitrogen to avoid enzymatic reactions during grinding, and then lyophilized overnight.

2.3 Spectrophotometric Analyses

2.3.1 Determination of Betalain Content

2.3.1 .1 Extraction of Betalains

A modified version of the method of Ravichandran et al. (2013) was used in extraction of betalains. Three batches of BVf and BVp are studied in three parallels of extraction. 1 g of freezedried samples were dissolved in 10 ml of 50% ethanol containing 50 mM of ascorbic acid/L (for pigment stability to avoid possible oxidations), were agitated for 10 s and the homogenate was centrifuged at 5000 rpm for 12 min. The supernatant was collected as it is after centrifugation and the same was repeated for two more times to ensure maximum extraction of betalains. The supernatant was further used for determination of betalains.

2.3.1.2 Quantification of Betalains

The quantification of betaxanthins and betacyanins in the extracts was determined spectrophotometrically at 538 nm and 480 nm, respectively. A UV–Vis spectrometer (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA). was used according to the methods of Stintzing, Schieber, and Carle (2003). The betalain concentrations were calculated using absorbances for each sample and expressed as the sum of red-violet and yellow-orange pigments. The betalain content (BC) was calculated as BC (mg/L) = [(A×DF×MW×1000) /(e×l)], where A is the absorption, DF the dilution factor and l the pathlength (1 cm) of the cuvette. The molecular weights (MW) and molar extinction coefficients (e) (MW=550 g/mol; e=60,000 L/mol cm in H2O) and (MW=308 g/mol; e=48,000 L/mol cm in H_2O) were applied for betacyanins and betaxanthins, respectively. Measurements were performed in duplicates.

2.3.2 Determination of Total Phenolic Content

2.3.2.1 Extraction of Phenolics

The phenolics in each sample was extracted according to Ravichandran et al. (2012) with slight modifications. 0.1 g of freeze dried samples were dissolved in 10 ml of 50% ethanol, were agitated for 10 s and the homogenate was centrifuged at 5000 rpm for 12 min. The supernatant was collected as it is after centrifugation and the same was repeated for 2 more times.

2.3.2.2 Folin-Ciocalteu Assay

Total phenolic content (TPC) were determined using Folin-Ciocalteau assay (Spanos and Wrolstad, 1990). 100 µl of diluted red beet flesh and peel samples extracted with 50% ethanol was mixed with 0.2 N 1.5 ml of Folin-Ciocalteu reagent and 1.2 ml of 7.5% of sodium carbonate. The mixture was allowed to stand for 90 minutes at room temperature in darkness. The absorbance was read at 765 nm using a microplate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA). A standard curve was plotted using gallic acid and the results were expressed on dry weight basis as mg of gallic acid equivalent (GAE) per 100 g of dry weight (DW) of sample. All samples were analyzed in triplicate.

2.3.3 Determination of Total Antioxidant Capacity

2.3.3 .1 DPPH Assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was used to measure the free–radical scavenging capacity of red beet (Kumaran et al., 2006; Rai et al., 2006). 100 µl of samples extracted with 50% ethanol was mixed with 2 ml of 0.1 mM DPPH in 100% methanol. After agitating 10 seconds, mixtures incubated at room temperature for 30 minutes in the dark and the absorbance was measured at 517 nm. Radical scavenging activity was calculated as the miligram Trolox equivalent (TE) values with respect to the standard for 100 g of dry weight.

2.3.3.2 CUPRAC Assay

CUPRAC (Cupric ion reducing antioxidant capacity) assay developed by Apak et al. (2005) was used. 100 μL of sample extract was mixed with 1 mL of 10 mM copper (II) chloride, 7.5 mM neocuproine and 1 M ammonium acetate buffer solution (pH:7). Immediately, 1 mL of distilled water was added to the mixture so as to make the final volume 4.1 mL. After 30 minutes of incubation at room temperature, absorbance was measured at 450 nm and then the results were calculated as mg of Trolox equivalent (TE) per 100 g DW of sample. Samples were analyzed in triplicate.

2.4. Analysis of Phenolics by UFLC-MS/MS

The analysis was performed using a Shimadzu 20A series ultra fast liquid chromatograph (UFLC, Shimadzu Coperation, Kyoto, Japan), a micro vacuum degasser (Prominence Degasser DGU-20A3, Schimadzu), an autosampler (Prominence AutoSampler SIL-20AHT, Schimadzu), a column oven (Prominence Column Oven CTO-10ASVP, Schimadzu), a controller (Prominence Controller CBM-20A Lite, Schimadzu) and an MS detector with electrospray ion source (ESI) and a triple quadrupole analyzer (API-3200 QTRAP, ABSciex, USA). The extraction was performed by the adopted method of Ravichandran et al. (2012) with slight modifications. A total of 1 gram of grounded dried samples was extracted for 15 min using 5 mL 70% methanol (v/v, pH 4, phosphoric acid) in an ultrasonic bath (USC900TH, VWR ultrasonic cleaner, Radnor, PA, USA) on ice. Samples were centrifuged for 12 min at 5000 rpm (Universal 32R, Hettich Zentrifugen, Tuttlingen, Germany). The supernatants were collected and the pellets were re-extracted twice more with 2.5 mL 70% methanol. The collected supernatants were finally filtrated using 0.22 μm filter, and then analyzed with LC/MS-MS. The choromatographic seperations were performed on an Inertsustain C18 column (150mm x 4,6mm, 3μm) with guard column (4. 0 x 10mm x 2). A gradient of mobile phase A (7.5 mM formic acid) and mobile phase B (acetonitrile) was used. The flow rate was 0.5 mL min-1 and the injection volume was 10 μL for each standard mixture and the column temperature was set to 40 °C. A 20 min gradient program was used with the gradient profile as follows: 0–1 min: 5% B, 1–2 min: 10% B, 2–5 min: 50% B, 5–10 min: 50% B, 10–15 min: 95% B, 15–19 min: 95% B, 19–20 min: 5% B. Identification and

quantification of phenolic acids present were done by comparing retention time and area of the peaks in the extracts with that of the standard phenolic acids (chlorogenic acid, caffeic acid, 4 (p-)Hydroxybenzoic Acid, trans-cinnamic acid, p-coumaric acid, ellagic acid, protocatechuic acid, ferulic acid, sinapic acid, syringic acid, rutin and gallic acid).

2.5 Simulation of In Vitro Gastrointestinal Digestion

The in vitro measurement of bioaccessibility is regarded to be a reliable tool to approach the bioavailability of dietary compounds, taking into account eventual variations owing to food source, digestive stability of the compound, and maximum solubility in the gastrointestinal medium, as an index of availability for eventual processes of apical uptake by absorptive epithelial cells (Tesoriere et al., 2008).

The in vitro gastrointestinal digestion model was a slightly modified version of the technique developed by McDougall et al. (2005) and Ortega et al. (2009) was performed in duplicate for three batches of beetroot fleshes and peels. The model comprises a three-step procedure in order to mimic the digestive process in the mouth, stomach (gastric digestion), and small intestine (duodenal digestion). ***Oral phase***. The digestion starts by adding 10 mg of α-amylase (100-300 ud/g) in 10 mL phosphate buffer solution (pH 6.9 with 0.1% NaCl) to 5 g of the BVf and BVp samples, which is incubated for 5 min in 250 mL beakers. ***Gastric phase.*** 10.5 mL gastric aliquots prepared by 1.5 mL pepsin solution (10 mg pepsin dissolved into 10 mL 0.1 N HCl) mixed with 10 mL distilled water was added. The samples in beakers were acidified to pH 1.7 with 5 N HCl and incubated at 37°C in a shaking water bath (Memmert, Schwabach, Germany) at 100 rpm for 2 hours. After gastric digestion, aliquots of the post-gastric (PG) digestion were collected from each sample. ***Intestinal phase.*** The pH was increased with the addition of 4.5 mL of pancreatin and bile salt mixture (18 mg pancreatin and 112.5 g bile salt dissolved into 4.5 mL distilled water) followed by the addition of dialysis bags filled with 25 mL (the amount required to neutralize the samples at pH 6.5) sodium bicarbonate (21 g $NaHCO_3$ dissolved in 500 mL distilled water). Samples were incubated again in a shaking water bath (100 rpm) at 37°C for another 2 hours to complete the intestinal phase of the *in vitro* digestion process. After the intestinal phase, the solutions in the

dialysis bags were taken as the IN sample representing the material that entered the serum, and the solution outside the dialysis bags were taken as the OUT sample representing material that remained in the gastrointestinal tract. Blank was also prepared with identical chemicals but without sample, and underwent the same conditions. The resultant samples are collected in eppendorf tubes and centrifuged at 4°C, 18000 rpm for 10 minutes before store at -20 °C until use. Later, the collected samples (PG, IN and OUT) were assayed for total phenolic content, antioxidant capacity and individual phenolic acids.

2.6. Statistical Analysis

The data obtained from three independent experiments was reported as mean ± standard deviation. Statistical analysis was carried out using SPSS software (version 22.0, SPSS, Chicago, IL, USA). Treatments were compared using one-way analysis of variance (ANOVA) followed by Tukey post hoc test, and $p < 0.05$ was considered significant. The correlation coefficients (R^2) were calculated using Microsoft Office Excel 2013 software (Microsoft Corporation, Redmond, WA, USA).

3. RESULTS AND DISCUSSION

3.1. Spectrophotometric Quantification of Betalains

The content of betaxanthins and betacyanins in the extracts was determined spectrophotometrically at 538 nm and 480 nm with a UV-Vis spectrometer (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA), respectively according to the methods of Stintzing, Schieber, and Carle (2003). The structural difference enables a simultaneous spectrophotometric measurement of the pigment content without the need of chromatographic or electrophoretic separation. The content of betalains differed greatly among cultivars as well as growing conditions (Lee et al., 2014). Analyses of betalain contents showed that there were substantial differences in both betalain concentrations and betacyanin/betaxanthin (BC/BX) ratios of BVf and BVp samples of three batches. The total betalain content was calculated by summing the average betacyanin and betaxanthin contents (Table 3.1). Peel contains higher amount of betalains than fleshes in correspondance with the literature. Kujala et al. (2000) reported a higher betacyanin betanin content (38.7 mg/g DW) in red beetroot peel than the red beetroot flesh (10.2 mg/g DW). Since there is no report on this variety of beetroot, the results are compared with the red beetroot studies. Similar to the observed results, Lee et al. (2014) studied nine different cultivars of red beetroot and the total betalain content ranged between 650-800 μg/g FW. For the studied variety, the betacyanin content of flesh is greater than the betaxanthin content whereas the peel contains higher amount of betaxanthin than betacyanin. Canadanovic-Brunet et al. (2011) reported total betacyanin content 24.18 mg/g DW and total betaxanthin content of 17.67 mg/g DW for ethanol extracts of red beet pomace whereas 18.78 mg/g DW betacyanin and 22.90 mg/g DW betaxanthin content for water extracts. They concluded that the ethanol extracts showed the highest betalain as well as the total phenolic content in comparison to the acetone and water extracts. It has been reported that both the extraction method and solvent

were noticeably affect the content of individual compounds in beetroot extracts (Castellanos-Santiago and Yahia, 2008; Kujala et al., 2001). Conclusively, as this variety is less pigmented than the red beetroot, the betalain content is lower than the reported values (Canadanovic-Brunet et al., 2011; Georgiev et al., 2010; Kujala et al., 2000).

Table 3.1: Betaxanthins, betacyanins and total betalain contents.

Sample	Betaxanthins	Betacyanins	Total betalains
BVf	0.3 ± 0.05	0.9 ± 0.01	1.2
BVp	1.6 ± 0.01	0.9 ± 0.01	2.5

The results are expressed as mg per g dry weight, given the mean for three replicates of three batches ± standard deviation.

3.2. Phenolic Content and Antioxidant Capacity

3.2.1 Total Phenolic Content

Phenolic acids are secondary metabolites that are commonly present in plant-derived foods. Among the variety of phenolic compounds, phenolic acids have attracted noticeable interest recently due to their many potential health benefits. They are powerful antioxidants and have been reported to demonstrate antibacterial, antiviral, anticarcinogenic, anti-inflammatory and vasodilatory actions (Breinholt, 1999; Duthie et al., 2000; Mattila and Hellström, 2006). Total soluble phenolic compounds (TPC) were determined using the Folin–Ciocalteu reagent and the values are expressed as equivalents of Gallic acid, which is the most commonly used standard in phenolic estimations since Gallic acid found to be more stable and pharmacologically active antioxidant. Besides, it has also been shown quantitatively to be equivalent to most other phenolics and give consistent and reproducible results (Sing et al., 2002; Sreramulu and Raghunath, 2010).

Kahkönen et al. (1999) indicated that the total phenolic contents of vegetables were very low compared to fruits. Beets had been reported as the highest total phenolic content, followed by red onion, broccoli and kidney beans (Vinson, Hap, Su, and

Zubik, 1998). There are several studies have reported on the relationships between phenolic content and antioxidant activity of foods. Some authors found a correlation between the phenolic content and the antioxidant activity, while others not. For instance, Velioglu et al. (1998) described a strong relationship between total phenolic content and antioxidant activity in selected fruits, vegetables and grain products. However, no correlation between phenolic content and antioxidant activity was shown in the study on total antioxidant activity and phenolic content of selected vegetables by Ismail, Marjan, & Foong (2004) as well as in the study on some plant extracts containing phenolic compounds by Kahkönen et al. (1999). These findings can be explained by the degree of variation between different phenolic compounds in their effectiveness as antioxidant (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999). Additionally, each type of plant tissue had a different antioxidant activity, contributed by different antioxidant components, such as vitamin C, α-tocopherol, β-carotene or phenolic compounds. An illustration to this fact is present in the data given by Kaur and Kapoor (2002) and Sreeramulu and Raghunath (2010) (Table 3.2 and Table 3.3). Also, the comparison of beets with other root vegetables in terms of antioxidant activity and phenolic content can be derived from these studies.

Table 3.2 : Antioxidant activity via β-carotene bleaching method and total phenolic content of ethanolic extracts of some root and tuber vegetables (Kaur and Kapoor, 2002).

Vegetables	Antioxidant activity (%)	Total phenolics (mg catechol/ 100g)
Beet root (*Beta vulgaris*)	73.3	323.0 ± 11.7
Black carrot (*Daucus carota*)	73.0	350.5 ± 12.9
Carrot (*Daucus carota*)	67.0	55.0 ± 0.9
Potato (*Solanum tuberosum*)	62.3	149.8 ± 6.3
Turnip (*Brassica rapa*)	47.8	127.0 ± 1.8
Radish (*Raphanus sativus*)	13.0	54.5 ± 0.8

Table 3.3 : Antioxidant activity and total phenolic content of some root and tuber vegetables on fresh basis. (Sreeramulu and Raghunath, 2010).

Vegetables	Total Phenolic Content[a]	Antioxidant activity	
		DPPH[b]	FRAP[c]
Red Beet (*Beta vulgaris*)	169.41 ± 40.19	125.10 ± 13.41	6308.09 ± 916.86
Carrot (*Daucus carota*)	22.21 ± 5.51	11.06 ± 3.03	256.31 ± 65.72
Potato (*Solanum tuberosum*)	38.42 ± 0.62	16.04 ± 8.20	704.73 ± 102.28
White Radish (*Raphanus sativus*)	66.73 ± 18.46	29.02 ± 5.20	1294.36 ± 188.68
Tapioca (*Manihot esculenta*)	137.55 ± 6.04	51.07 ± 7.10	3002.40 ± 72.17
Yam (*Typhonium trilobatum*)	54.92 ± 8.15	74.05 ± 10.20	2891.47 ± 310.24

[a] Total Phenolic content expressed in mg GAE/100g FW
[b]DPPH: Free Radical Scavenging Activity mg Trolox equivalents/100g FW
[c]FRAP: Ferric Reducing Antioxidant Power mg $FeSO_4$ equivalents/ 100g FW

Phenolic compounds are known to play a role in plant defense mechanisms as well as in the antioxidant activity. However, the effect of polyphenols strongly depends on the type of polyphenol and its combination with other compounds (Bavec et al., 2010). Data given in Table 3.4 summarizes the total phenolic contents of BVf and BVp samples. The standard deviations are quite high in terms of antioxidant capacities as well as phenolic content. It is because of the differences between the batches that result from the variations of samples with respect to time of harvest and location of farms. The total polyphenol content of BVf and BVp samples followed the order as BVp> BVf in consistence with the findings of Kujala et al. (2000). They founded that the TPC of red beet samples decreases in the order peel>crown>flesh. Values measured in their studies ranged from 15.5 mg GAE/g DW in the peel to 4.2 mg GAE/g DW in the flesh. In another study, Kahkönen et al. (1999) reported lower values for the total phenolics in red beet peel and sugar beet peel as 4.3 mg GAE /g DW and 4.2 mg GAE /g DW, respectively. Moreover, Bavec et al. (2010) determined the TPC content of Beta vulgaris L. ssp. vulgaris Rote Kugel between 3.16-4.94 mg GAE /g DW. Values of 14.6 ± 0.5 mg GAE g^{-1} have been reported for Tunusian red beetroots (Ben Haj Koubaier et al., 2013). On the other hand, Canadonovic et al. (2011) and Georgiev et al. (2010) reported greater amounts of phenolic contents for red beetroot pomace and intact plants, 376.4 mg GAE/ g DW

and 218.3 mg GAE/ g DW, respectively. When we compare these findings with regards to the per gram values determined in this study, where values of TPC lie between 7.55-8.22 mg GAE/ g DW (BVp) and 5.13-5.88 mg GAE /g DW (BVf), results are in a similar range for this beetroot variety to that in previous findings for red beetroots (Bavec et al., 2010; Kahkönen et al., 1999; Kujala et al., 2000) and substantially higher than reported for sugar beet pulp phenolics, 1.79 mg GAE/ g DW (Mohdaly et al., 2010).

Table 3.4 : Average phenolic contents and total antioxidant capacities on dry basis. Means of triplicated analyses are given with two significant digits and ± standard deviations.

Sample	TPC[a]	DPPH[b]	CUPRAC[c]
BVf	545,43 ± 38,55	62,94 ± 13,80	836,34 ± 97,46
BVp	850,41 ± 33,58	112,90 ± 31,12	1898,36 ± 168,13

[a] TPC: Total phenolic content expressed in mg GAE/100g DW
[b]DPPH: Free Radical Scavenging Activity mg TEAC/100g DW
[c]CUPRAC: Cupric Reducing Antioxidant Capacity mg TEAC/ 100g DW).

3.2.2 Antioxidant Capacity

An antioxidant can be defined as substance that opposes oxidation and protecting cells from the damage. Dietary antioxidants, including polyphenolic compounds, are believed to be the effective nutrients in the prevention of these oxidative stress related diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders, and certain types of cancer (Huang et al., 2005). Since there are many assays which differ from each other in terms of reaction mechanisms, oxidant and target/probe species, reaction conditions, and expression of results have been developed and tested in the literature, it is important to quantify the total antioxidant capacity using more than one method in order to reach reliable results (Karadag, Ozcelik and Saner, 2009).

The antioxidant capacity of samples was determined using two different assays, namely DPPH and CUPRAC. The method of scavenging the stable DPPH radical is widely used to evaluate antioxidant activities in a relatively short time compared with other methods (Duh et al. 1999; Chang et al. 2002). The effect of antioxidants on DPPH radical scavenging was thought to arise from their hydrogen donating ability. DPPH radical is a stable free radical and accepts an electron or hydrogen radical to become a stable molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. It is also visually noticeable as a discoloration from purple to yellow. Cupric ion reducing antioxidant capacity (CUPRAC) assay is also a spectrophotometric method, which can simultaneously measure hydrophilic and lipophilic antioxidants at physiological pH as the reagent used, neocupreine, is soluble both in water and organic media (Apak et al., 2005). Figure 3.1 shows the antioxidant capacities of flesh and peel samples determined by DPPH radical scavenging method as well as CUPRAC method expressed as mg Trolox equivalents.

In the literature, broad ranges in antioxidant activities have been reported previously for the same material (Cao et al., 1996; Gazzani et al., 1998; Kaur and Kapoor, 2002; Morales-Soto et al., 2014). This could be due to factors like agronomic, genomic and post harvesting conditions which may influence the chemical composition of plant foods (Imeh & Khokhar, 2002; Kahkönen et al., 1999). The presence of different antioxidant compounds in fruits and vegetables possess difficulties to measure each antioxidant component separately. Therefore, several methods are used to calculate the total antioxidant capacity of biological samples as well as different extraction mediums to provide the maximum extraction of the available antioxidants from the samples (Gazzani et al., 1998; Velioglu et al., 1998; Vinson et al., 1998; Kahkonen et al., 1999; Kaur and Kapoor, 2002; Morales-Soto et al., 2014). In the present study, aqueous ethanol extraction was used since it has been reported to be more efficient than water in extracting the antioxidants and pigments present in the vegetables (Kaur and Kapoor, 2002; Azeredo, 2006).

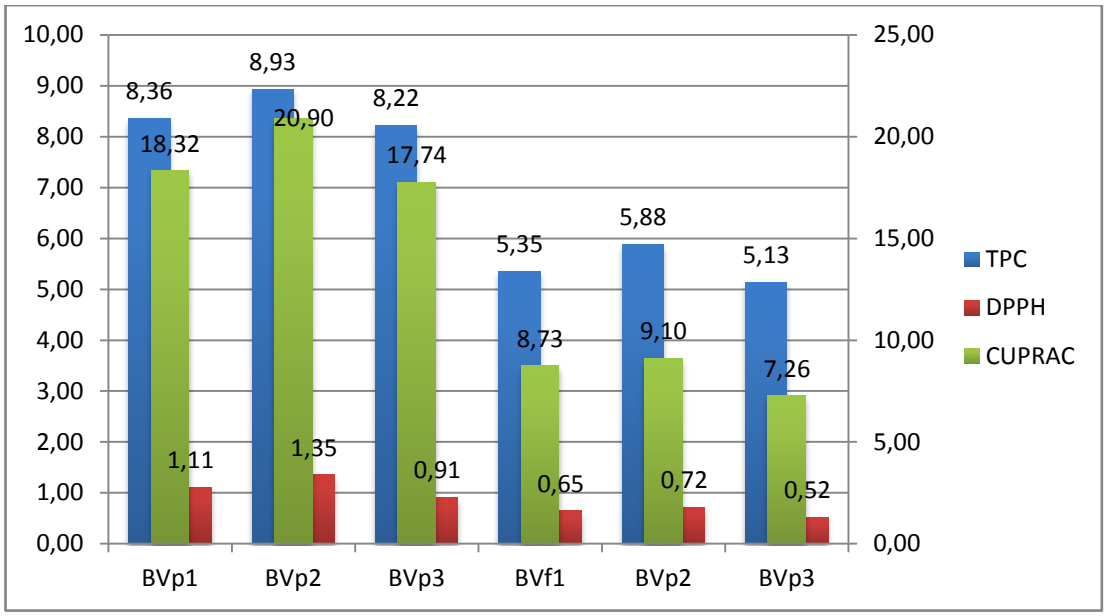


Figure 3.1 : Comparison of total phenolic contents and total antioxidant capacities of different batches. Results are means of three determinations.

TPC: Total Phenolic Content determined by Folin-Ciocalteu method (calculated as mg GAE/ g DW) DPPH: Free Radical Scavenging Activity (calculated as mg TEAC/ g DW) CUPRAC: Cupric Reducing Antioxidant Capacity (calculated as mg TEAC/ g DW).

An obvious conclusion can be derived from these results is that the peels have greater antioxidant capacities with greater content of phenolics than the fleshes. The difference between three batches of peel and flesh samples were statistically significant ($p < 0.05$) for the three assays performed. According to the results, the total phenolic contents of samples varied as BVp1>BVp2>BVp3>BVf2>BVf1=BVf3 whereas the free radical scavenging capacity of samples varied as BVp1>BVp2>BVp3=BVf2=BVf1=BVf3 and the total antioxidant capacities varied as BVp2>BVp1=BVp3>BVf3=BVf2>=BVf1. The order slightly changes between batches with respect to different methods. As it has been discussed in the previous part, although there are studies correlating the phenolic content and the antioxidant activity of plant tissues, several studies exist in the literature indicating zero correlation. Mariko, Hassimotto, Genovese, & Lajola, 2005 suggested that this lack of correlation could be owing to the distinct antioxidant activity parameters determined in those studies and/or different responses of phenolic compounds in different antioxidant activity assay systems (Kahkonen et al., 1999). Furthermore, each type of plant tissue had a different antioxidant activity,

contributed by different antioxidant components, such as vitamin C, α-tocopherol, as well as pigment substances such as β-carotene, anthocyanin in addition to the phenolic compounds. For the analyzed beetroot variety in this study, the contributing antioxidant component supposed to be betalain pigments.

In the literature, several total antioxidant capacity methods were reported for red beetroot. However, to our knowledge, no report exist using Cupric Reducing Antioxidant Capacity assay. Sreeramulu and Raghunath (2010) founded the DPPH free radical scavenging capacity as 125.10 ± 13.41 mg TEAC/ 100g FW which is two folds higher than our outcome. Similarly, Georgiev et al. (2010) reported 14.2% ± 0.7% inhibition for red beet as a result of DPPH assay which is also greater than our inhibition, 5.05% ± 0.9% inhibition for flesh samples. On the other hand, authors reported quite higher values of total antioxidant capacity using other assays than the DPPH assay in correspondence with our results. Similarly, the CUPRAC values for the BVf samples in this study are approximately 15 folds higher than the DPPH values. Ravichandran et al. (2012) founded 5 folds higher values using ABTS (2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) assay than the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay for red beetroot. Moreover, Sreeramulu and Raghunath (2010) reported approximately 50 folds higher values using FRAP (Ferric Reducing Antioxidant Capacity) assay for red beet. It has been reported that factors such as the stereoselectivity of radicals and the solubility of extracts in different test systems affect the capacity of extracts to react with and quench different radicals (Yu et al., 2002). Similarly, scavenging of the ABTS+ radical by sugar beet pulp extracts was found to be higher than that of the DPPH radical (Mohdaly et al., 2010). Besides, Wang et al. (1998) reported that some compounds possessing ABTS+ radical scavenging activity did not show DPPH radical scavenging activity.

3.3. Correlation between Spectrophotometric Assays

The linear correlation coefficients (R^2), calculated between the results obtained for TBC, TPC, and TAC assays, were given in Table 3.5. The correlations between CUPRAC-TBC (R^2=0.942) and CUPRAC-TPC (R^2=0.992) were higher in

comparison to the values obtained for the correlations of the DPPH assay with TBC/TPC. The lowest correlation was observed in between the TBC and DPPH assay results (R^2=0.726). The correlation coefficients (R^2) calculated between the results of the applied spectrophotometric methods indicated the highest correlation between TPC and CUPRAC assay results (R^2=0.997). In addition, CUPRAC assay gave high correlations with TBC and TPC whereas DPPH assay gave low correlations with them.

Table 3.5: Correlation coefficients (R^2 values) between TBC, TPC, and TAC assay results.

	TBC	**TPC**	**DPPH**	**CUPRAC**
TBC	1	-	-	-
TPC	0,947*	1	-	-
DPPH	0,726*	0,885*	1	-
CUPRAC	0,942*	0,992*	0,902*	1

* Significant difference between mean values of BVp and BVf samples at P< 0.05.
Abbreviations: TBC (Total Betalain Content); TPC (Total Phenolic Content); DPPH (2,2-diphenylpicrylhydrazyl Radical Scavenging Activity); CUPRAC (Cupric Reducing Antioxidant Capacity)

The good linear relationships between CUPRAC assay and the other spectrophotometric assays, TBC and TPC, may attributed to this method's ability to react with a variety of antioxidant compounds, regardless of chemical type or hydrophilicity, making it the most consistent method of total antioxidant measurement in relation to Folin reagent-responsive TPC (Apak et al., 2006). On the other hand, lower total antioxidant capacity values were reported using DPPH assay in comparison to other methods such as ABTS and FRAP in the literature (Sreeramulu and Raghunath, 2010; Ravichandran et al., 2012). Factors such as the stereoselectivity of radicals and the solubility of extracts in different test systems affect the capacity of extracts to react with and quench different radicals (Yu et al., 2002). This could explain the relatively lower correlations observed between DPPH assay and the other assays performed.

3.4. Phenolics Profile by UFLC-MS/MS

The qualitative and quantitative analyses of polyphenolic compounds were performed by UFLC/MS-MS system which is equipped with an ultra fast liquid chromatography (Prominence Liguid Chromatograph LC-20AD, Schimadzu). Identification and quantification of phenolic acids present were done by comparing retention time and area of the peaks in the extracts with that of the standard phenolic acids (chlorogenic acid, caffeic acid, 4 (p-)Hydroxybenzoic Acid, trans-cinnamic acid, p-coumaric acid, ellagic acid, protocatechuic acid, ferulic acid, sinapic acid, syringic acid, rutin and gallic acid).

Phenolic compounds have been correlated with health benefits due to their antioxidant activity (Velioglu, Mazza, Gao, & Oomah, 1998). With the scope of this study, the phenolic composition of this unknown beetroot variety is described for the first time. Five major phenolics were determined by UFLC-MS/MS system. Similar chromatogram patterns of phenolic acids were observed for peel and flesh samples however, their contents differed depending on batches. From the results in Table 3.6 and Table 3.7, it was noticed that chlorogenic acid and ferulic acid was the major constituents for peel samples whereas ferulic acid was the only major phenolic in flesh samples followed by syringic, vanillic and 4 (p-)hydroxybenzoic acid. However, caffeic acid and rutin were present only in trace amounts. Ellagic acid, procatechuic acid, gallic acid, sinapic acid, trans-cinnamic acid and *p*-coumaric acids were not detected.

Table 3.6: UFLC/MS-MS results for phenolic acid contents of BVf samples.

Phenolic acid	Retention Time			BVf		
	1.batch	2.batch	3.batch	1.batch	2.batch	3.batch
4 (p-)Hydroxybenzoic Acid	8,35	8,36	8,36	646,50	108,60	150,10
Vanilic Acid	8,41	8,43	8,42	290,30	205,30	228,0
Ferulic Acid	8,77	8,79	8,78	7380,0	9563,0	2233,0
Syringic Acid	8,30	8,31	8,30	1246,0	836,0	699,60
Chlorogenic Acid	7,85	7,87	7,86	59,50	47,70	45,40
∑				9622,3	10760,6	3356,1

BVf: Flesh samples. Retention time is given in min. Results are expressed as μg per g dry weight (n=3).

Table 3.7: UFLC/MS-MS results for phenolic acid contents of BVp samples.

Phenolic acid	Retention Time			BVp		
	1.batch	2.batch	3.batch	1.batch	2.batch	3.batch
4 (p-)Hydroxybenzoic Acid	8,34	8,35	8,35	2766,0	1790,0	1123,0
Vanilic Acid	8,42	8,43	8,41	519,0	673,0	1150,0
Ferulic Acid	8,79	8,79	8,78	6450,0	3453,0	2550,0
Syringic Acid	8,30	8,30	8,30	2973,0	4223,0	2633,0
Chlorogenic Acid	7,86	7,87	7,87	7063,0	1166,0	44,4
∑				19771	11305	7500,4

BVp: Peel samples. Retention time is given in min. Results are expressed as µg per g dry weight (n=3).

Although, there were no differences in the phenolic profiles of the extracts, the peels had higher concentrations of the identified phenolic compounds than the fleshes. It is in accordance with the results obtained from Folin-Ciocalteu assay. Ferulic acid was the major constituent in both BVf and BVp samples. It is considerable that chlorogenic acid was present substantially higher amount in peel samples than the fleshes.

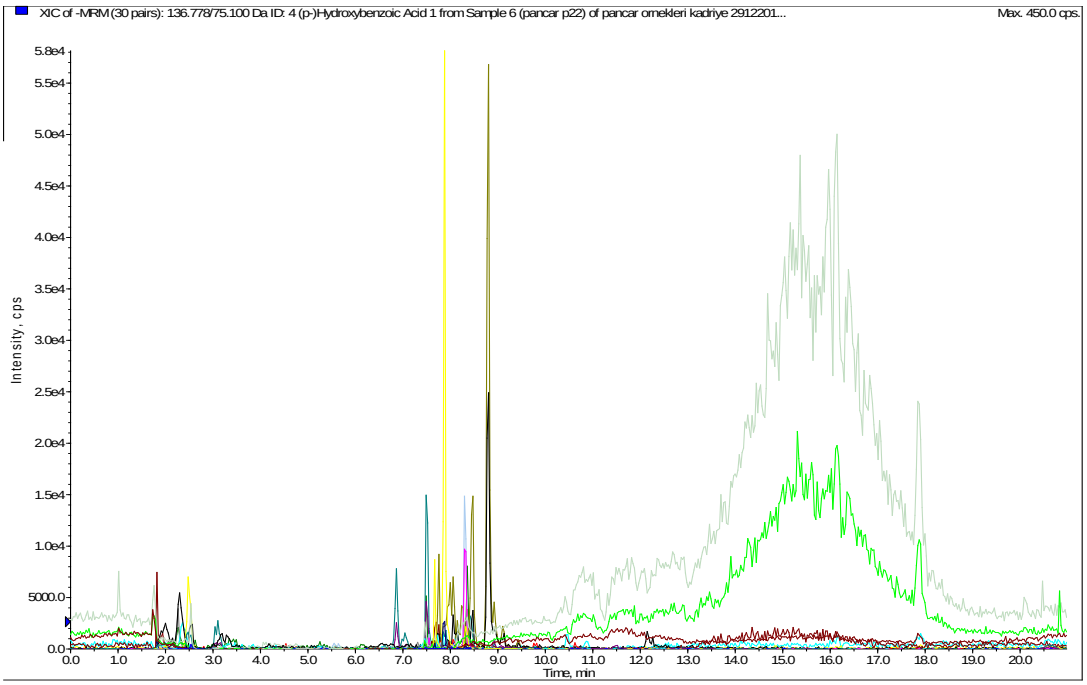


Figure 3.2: Peel chromatogram of phenolic acids by UFLC/MS-MS.

Similar chromatogram patterns of phenolic acids were observed for peel and flesh samples however, contents differed depending on batches ($p<0.05$). The order of individual phenolic acid contents were as follows: ferulic acid > chlorogenic acid > syringic acid > 4-hydroxybenzoic acid > vanilic acid for peels and ferulic acid > syringic acid > vanilic acid > 4-hydroxybenzoic acid > chlorogenic acid for fleshes.

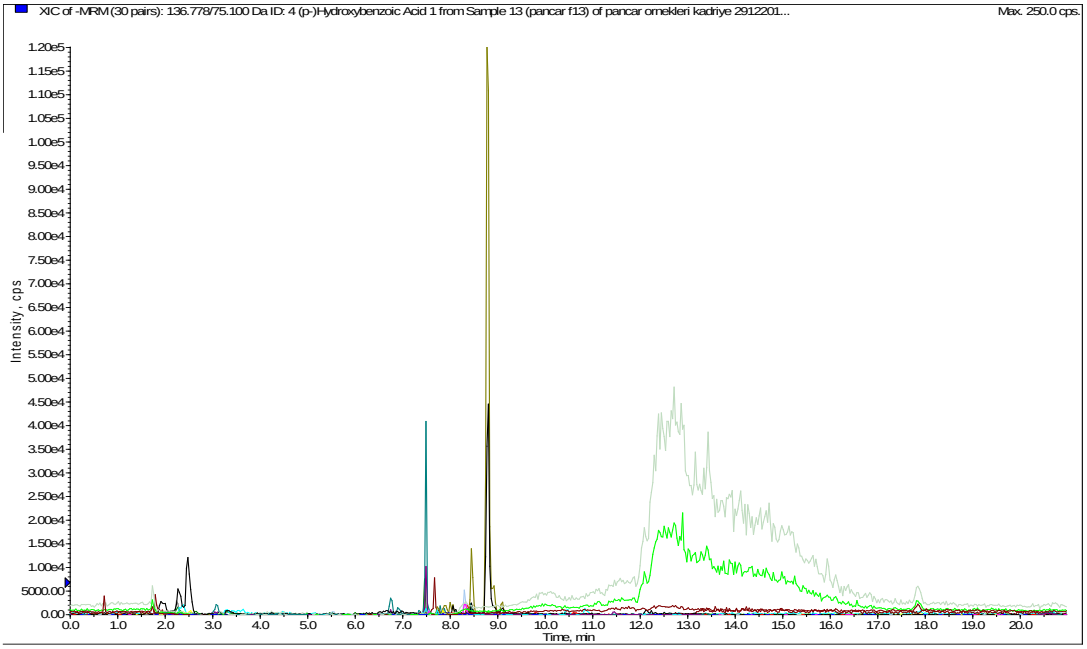


Figure 3.3. Flesh chromatogram of phenolic acids by UFLC/MS-MS.

On the contrary, 4-hydroxy benzoic acid was reported as the major phenolic constituent in red beet followed by cinnamic acid, vanillic, chlorogenic, trans ferulic acid, caffeic acid, and epicatechin. Coumaric and anis acids were founded only in trace amounts (Georgiev et al., 2010; Kujala et al., 2000; Ravichandran et al., 2012). Ellagic acid was only detected in the root acetonitrile soluble fraction (Ben Haj Koubaier et al., 2013). Conclusively, red beet has been reported more phenolics content qualitatively and quantitatively. Moreover, Obata et al. (1963) determined the phenolic acids in sugar beet molasses as catechol, p-hydroxybenzoic acid, 2-hydroxydihydrocinnamic acid, salicylic acid, syringic acid, vanillin and vanillic acid.

Osorio-Esquivel et al. (2011) studied phenolics of sour prickly pear (Opuntia joconostle) that is a kind of cactus fruit grown in Mexico. It a betalain containing plant but not as high as red beetroots. They identified phenolic acids of procatechuic

acid, caffeic acid, 4-hydroxybenzoic acid, and syringic acid with the descending order of concentration. This profile seems more similar with the studied variety of beetroot than the reported phenolic acids for red beets.

3.5 In Vitro GI Digestion

In recent years, bioaccessibility studies have given evidence of the absorption of any phytochemicals and have shown that uptake varies widely, depending on the type of compound and/or food matrix (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). In vivo studies on humans and animals are not preferred due to the limitations of complexity, expensiveness and ethical reasons (Guven et al., 2010). On the other hand, in vitro digestion method is a rapid and simple to evaluate phytochemical stability in proceed food (McDougall et al., 2005a).

3.5.1 Total Phenolics after GI Digestion

Results for total phenolic content analysis after GI digestion were expressed as mg gallic acid equivalents per g standard for each GI digestion samples obtained from three fractions (PG, IN and OUT). The standard calibration curve of gallic acid shown in Figure 3.4 was prepared in concentrations between 0.005-0.1 mg/ml and obtained equation from the calibration curve was used to calculate the gallic acid equivalent values of the samples measured spectrophotometrically.

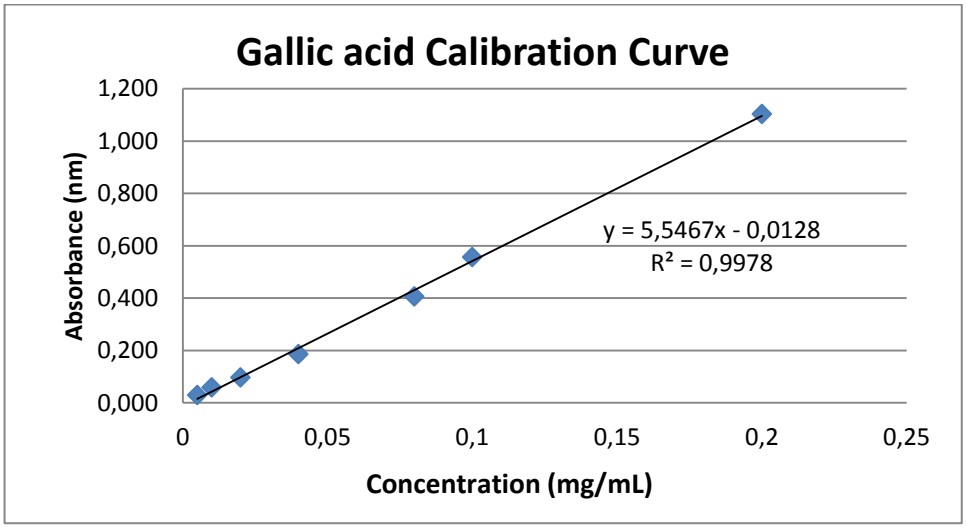


Figure 3.4: Calibration curve of Gallic acid standard in water for Folin-Ciocalteu assay (IN, OUT, PG fractions).

Generally, the total phenolic contents of PG fractions were higher than the values for OUT and IN fractions. It is because of the acidity of gastric medium stabilizes the polyphenolic compounds and favor their release from the food matrix. On the contrary, several phenolic acids degraded at the mild alkaline pH of the small intestine (Tagliazucchi et al., 2010). During gastro-intestinal digestion, polyphenols may either interact with other food constituents such as sugars, lipids and fibre, that causes reduction or improvement in their bioaccessibility (Palafox-Carlos, Ayala-Zavala, & Ganzalez-Aguilar, 2011; Toydemir et al., 2013).

Table 3.8 : The total phenolic content of the flesh and peel extracts before and after in vitro GI digestion.

Sample		INITIAL	PG	IN	OUT
BVf	1.batch	5.35 ± 0.10	1.49 ± 0.01	0.74 ± 0.02	0.70 ± 0.03
	2.batch	5.88 ± 0.25	1.81 ± 0.02	0.88 ± 0.04	0.97 ± 0.27
	3.batch	5.13 ± 0.12	1.74 ± 0.02	0.60 ± 0.03	0.52 ± 0.03
	Mean	5.45 ± 0.27	1.68 ± 0.15	0.74 ± 0.13	0.73 ± 0.23
BVp	1.batch	8.36 ± 0.18	1.99 ± 0.03	0.89 ± 0.08	1.78 ± 0.00
	2.batch	8.93 ± 0.22	2.10 ± 0.02	0.84 ± 0.10	1.52 ± 0.39
	3.batch	8.22 ± 0.06	1.77 ± 0.02	1.16 ± 0.02	1.37 ± 0.02
	Mean	7.89 ± 0.29	1.95 ± 0.15	0.97 ± 0.17	1.56 ± 0.26

BVf: Flesh samples; BVp: Peel samples; INITIAL: TPC of the sample before digestion; PG: TPC of the sample after digestion in stomach; IN: TPC of the sample after digestion in small intestines. OUT: TPC of the sample that can not be recovered by GI tract. All values are given as mean values of triplicate runs with its standard deviations. All values are expressed as mg Gallic acid equivalents per g dry weight.

The results of the bioaccessibility for total phenolic contents are given in Table 3.8. The anaylsis of total phenolic content of digested samples showed 3 to 9 fold decrease of TPC in comparison with the raw material (INITIAL). The order of total phenolic content is as PG > OUT > IN fractions in accordance with the literature (Bouayed et al., 2012; Liang et al., 2012; Tagliazucchi et al., 2010; Tavares et al., 2012).

The reduction of phenolic content in peel samples is greater than in fleshes and statistically significant ($p<0.05$). It may be attributed to the presence of other nutrients in the flesh part known as interfering substances in Folin-Ciocalteu assay (Prior et al., 2005), such as ascorbic acid, sugars (fructose and sucrose), aromatic amines, sulfur dioxide, and organic acids. Root crops of the species Beta vulgaris store its excess energy as sugar and not as carbohydrate polymers. The sugar is primarily stored in the form of sucrose, and constitutes more than 99% of the total sugar (Bach, 2012). Bach (2012) studied distinct varieties of beetroot that are Taunus (red beet), Pablo (red beet), Touchstone Gold (yellow beet), and Chioggia (candy-striped beet) (Figure 1.3) and determined that candy-striped beet has the highest content of total sugar (8.2 g per 100 g tuber) among other beetroot varieties. The reported red beets contain 7.2 g per 100 g tuber averagely whereas the yellow beet founded as 7.4 g per 100 g tuber.

In order to get more precise data, correction for the interfering substances should be made, or alternatively the effect of such compounds could be minimized by using solid-phase extraction before analysis (George et al., 2005). The differences in bioaccessibility of polyphenolics between the batches were also statistically significant ($p<0.05$). However, it is not correlated between peel and flesh samples.

Percent recovery of total phenolic contents obtained by Folin-Ciocalteu assay with respect to the GI digestion fractions (IN, OUT and PG) by assuming the initial values as 100%. The results are summarized in Table 3.9.

Table 3.9: Total phenolic content recoveries % for PG, IN and OUT fractions.

Sample		**INITIAL**	**PG**	**IN**	**OUT**
BVf	1.batch	100	27.8	13.8	13.1
	2.batch	100	30.8	14.9	16.4
	3.batch	100	33.9	11.6	10.1
	Mean	100	30.8	13.5	13.2
BVp	1.batch	100	23.8	10.6	21.2
	2.batch	100	35.7	9.4	17.0

3.batch	100	34.5	14.1	16.6
Mean	100	33.0	11.3	19.8

BVf: Flesh samples; BVp: Peel samples; INITIAL: TPC of the sample before digestion; PG: TPC of the sample after digestion in stomach; IN: TPC of the sample after digestion in small intestines. OUT: TPC of the sample that can not be recovered by GI tract. All values are given as mean values of triplicate runs.

3.5.2 Total Antioxidant Capacities After GI Digestion

The antioxidant capacity was determined by DPPH method in Bvf and BVp samples collected from fractions (PG, IN and OUT) after in vitro GI digestion. Standard calibration curve was prepared by using Trolox and it is given in Figure 3.5. The results were expressed as mg TEAC/g standard. The standard calibration curve was prepared between 0.01- 0.4 mg/ml concentrations and the equation obtained from the curve was used to calculate the antioxidant activity of the sample measured spectrophotometrically. The results are shown in Table 3.10.

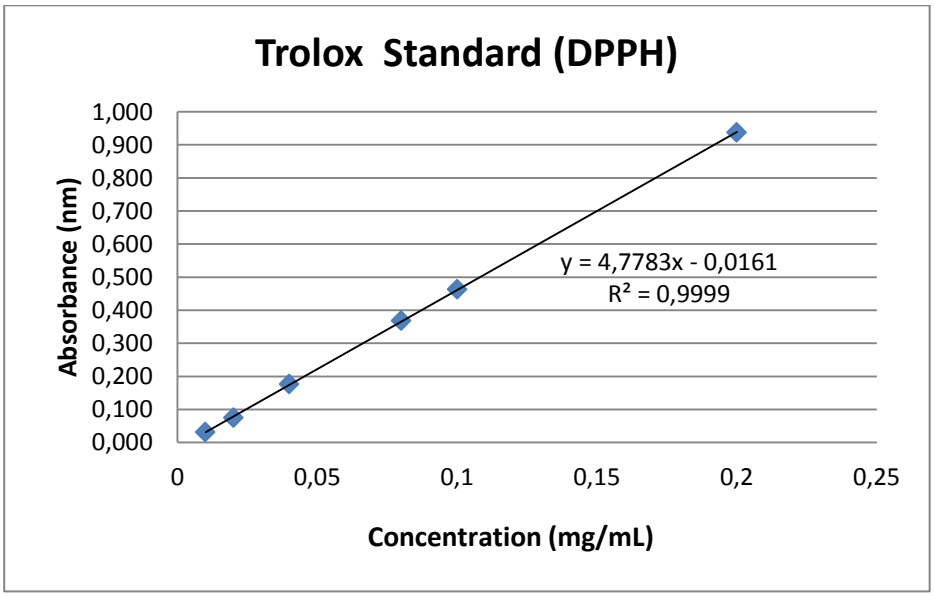


Figure 3.5: Calibration curve of Trolox standard in water for DPPH assay (IN, OUT, PG fractions).

Table 3.10 : Total antioxidant capacity by DPPH assay of the flesh and peel extracts
before and after in vitro GI digestion.

Sample		INITIAL	PG	IN	OUT
BVf	1.batch	0.65 ± 0.15	0.85 ± 0.02	n.d.	n.d.
	2.batch	0.72 ± 0.20	0.75 ± 0.00	n.d.	n.d.
	3.batch	0.52 ± 0.02	0.67 ± 0.04	n.d.	n.d.
	Mean	0.57 ± 0.02	0.99 ± 0.14		
BVp	1.batch	1.11 ± 0.17	1.16 ± 0.03	n.d.	n.d.
	2.batch	1.35 ± 0.20	0.96 ± 0.04	n.d.	n.d.
	3.batch	0.91 ± 0.40	0.85 ± 0.03	n.d.	n.d.
	Mean	1.43 ± 0.49	0.76 ± 0.76		

BVf: Flesh samples; BVp: Peel samples; INITIAL: 2,2-diphenyl-1-picrylhydrazyl radical scavenging antioxidant capacity of the sample before digestion; PG: 2,2-diphenyl-1-picrylhydrazyl radical scavenging antioxidant capacity of the sample after digestion in stomach; IN: 2,2-diphenyl-1-picrylhydrazyl radical scavenging antioxidant capacity of the sample after digestion in small intestines. OUT: 2,2-diphenyl-1-picrylhydrazyl radical scavenging antioxidant capacity of the sample that can not be recovered by GI tract. n.d.: Not detected. All values are given as mean values of triplicate runs with its standard deviations. All values are expressed as mg Trolox equivalents per g dry weight.

In relation to the results of TPC analysis, the non-detected antioxidant capacity by DPPH assay in IN, OUT fractions is quite noticeable. However, as it has been dicussed previously, many authors reported significantly lower values for DPPH assay than other methods. According to the CUPRAC assay results, the recovery of total antioxidant capacity for BVf and BVp samples ranged betweeen 5.2% and 7.6% for IN fraction whereas considerably higher IN values were obtained the recovery of TPC (12.1-13.5 %). This result brings the question of interferences in Folin-Ciocalteu assay. It has been known that the presence of nutrients such as sugars lead to interference in Folin-Ciocalteu assay (Prior et al., 2005). Beetroots are rich sources of sugars in particular sucrose as the root store its excess energy in this form instead of carbohydrate polymers (Bach, 2012). The beetroot sample studied in the scope of this work is the most similar variety with the candy-stiped beet that has been reported as the highest total sugar content among other red beet and yellow beet varieties

(Bach, 2012). Therefore, the slightly lower recovery of antioxidant capacity in spite of the obtained TPC recovery values could be attributed to the high sugar content of beet samples. Because, the reported recoveries of polyphenols in the literature are mostly proportional with the recoveries of antioxidant capacity in food samples (Bouayed et al., 2012; Liang et al., 2012; Tagliazucchi et al., 2010; Tavares et al., 2012).

The recovery rates referring to the DPPH assay before and after digestion in GI tract are represented in Table 3.11.

Table 3.11. Total antioxidant capacity (DPPH assay) recoveries % for PG, IN and OUT fractions.

Sample		INITIAL	PG	IN	OUT
BVf	1.batch	100	130.7	n.d.	n.d.
	2.batch	100	104.2	n.d.	n.d.
	3.batch	100	128.8	n.d.	n.d.
	Mean	100	121.2		
BVp	1.batch	100	104.5	n.d.	n.d.
	2.batch	100	71.1	n.d.	n.d.
	3.batch	100	93.4	n.d.	n.d.
	Mean	100	89.9		

BVf: Flesh samples; BVp: Peel samples; INITIAL: 2,2-diphenyl-1-picrylhydrazyl radical scavenging antioxidant capacity of the sample before digestion; PG: 2,2-diphenyl-1-picrylhydrazyl radical scavenging antioxidant capacity of the sample after digestion in stomach; IN: 2,2-diphenyl-1-picrylhydrazyl radical scavenging antioxidant capacity of the sample after digestion in small intestines. OUT: 2,2-diphenyl-1-picrylhydrazyl radical scavenging antioxidant capacity of the sample that can not be recovered by GI tract. n.d.: Not detected. All values are given as mean values of triplicate runs.

Trolox standard calibration curve was prepared for CUPRAC method as shown in Figure 3.6 and the results were expressed as mg Trolox equivalents per g standard. The standard calibration curve was prepared between 0.01-0.4 mg/ml and the

equation was used to calculate the antioxidant activity of the samples measured spectrophotometrically. The results are shown in Table 3.12.

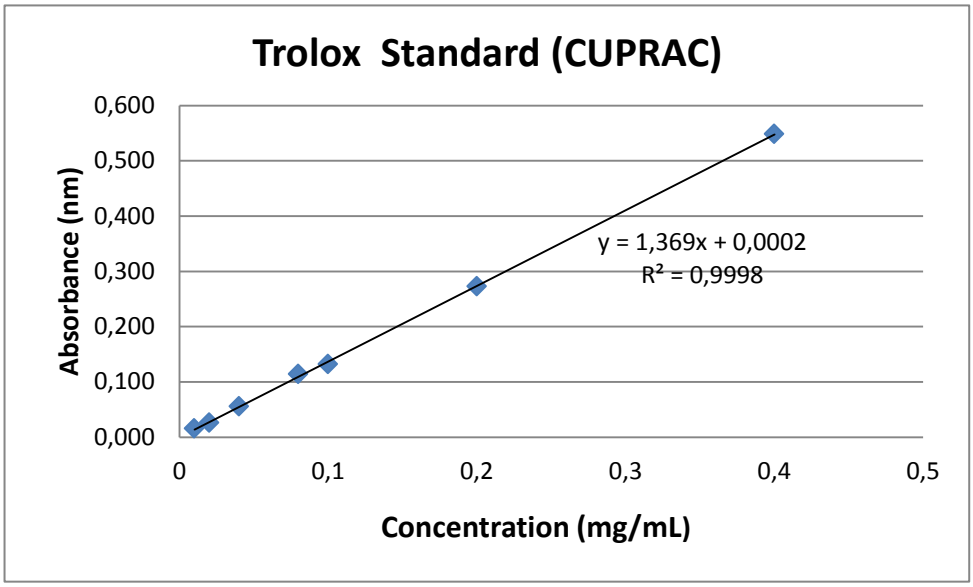


Figure 3.6: Calibration curve of Trolox standard in water for CUPRAC assay (IN, OUT, PG fractions).

The change in the antioxidant capacity via CUPRAC assay after in vitro GI digestion was found statistically insignificant for the BVf samples ($p<0.05$), but was significant for the BVp samples ($p>0.05$) in all fractions, PG, OUT, and IN. However, according to the DPPH assay, the change in antioxidant capacity was significant for both the BVf and BVp samples ($p>0.05$). Taking everything into consideration, the recovery rates of the bioavalability for TPC and CUPRAC indicated that beetroot flesh could be more efficiently digested than the peel samples since they all had a higher recovery range.

Results of total antioxidant capacity by CUPRAC assay of the flesh and peel extracts before and after in vitro GI digestion and the recovery rates are represented in Table 3.12.

Table 3.12 : Total antioxidant capacity by CUPRAC assay of the flesh and peel extracts before and after in vitro GI digestion.

Sample		INITIAL	PG	IN	OUT
BVf	1.batch	8.73 ± 0.40	1.50 ± 0.03	0.66 ± 0.05	2.62 ± 0.08
	2.batch	9.10 ± 0.40	1.51 ± 1.63	0.81 ± 0.08	2.90 ± 0.06
	3.batch	7.26 ± 2.04	1.97 ± 0.05	0.47 ± 0.03	3.43 ± 0.11
	Mean	8.36 ± 1.01	1.54 ± 0.41	0.65 ± 0.16	2.98 ±0.69
BVp	1.batch	18.32 ± 0.26	1.33 ± 0.03	1.12 ± 0.13	1.87 ± 0.08
	2.batch	20.90 ± 0.53	3.00 ± 0.08	0.98 ± 0.22	3.14 ± 0.06
	3.batch	17.74 ± 0.53	0.77 ± 0.07	0.86 ± 0.05	2.66 ± 0.08
	Mean	18.98 ± 1.51	1.71 ± 1.04	0.99 ± 0.17	2.56 ± 0.58

BVf: Flesh samples; BVp: Peel samples; INITIAL: Cupring reducing antioxidant capacity of the sample before digestion; PG: Cupring reducing antioxidant capacity of the sample after digestion in stomach; IN: Cupring reducing antioxidant capacity of the sample after digestion in small intestines. OUT: Cupring reducing antioxidant capacity of the sample that can not be recovered by GI tract. All values are given as mean values of triplicate runs with its standard deviations. All values are expressed as mg Trolox equivalents per g dry weight.

Theoretically, alterations that occurred in the structure of antioxidants during digestion may affect their reactivity with the less biologically relevant nitrogen radical formed in the DPPH assay, leading to underestimation of TAC after gastric phase (Wootton-Beard et al., 2011). Similar mechanisms may possibly contribute to explaining the differences observed between the measurements of TAC in the DPPH and CUPRAC assays after the intestinal phase. The recovery rates referring to the CUPRAC assay before and after digestion in GI tract are represented in Table 3.13.

Table 3.13. Total antioxidant capacity (CUPRAC assay) recoveries % for PG, IN and OUT fractions.

Sample		INITIAL	PG	IN	OUT
BVf	1.batch	100	17.2	7.6	30.0
	2.batch	100	16.6	8.9	20.9
	3.batch	100	27.1	6.5	31.8

	Mean	100	20.3	7.6	27.5
BVp	1.batch	100	7.3	6.1	10.2
	2.batch	100	14.3	4.7	15.0
	3.batch	100	4.4	4.8	14.9
	Mean	100	8.7	5.2	13.3

BVf: Flesh samples; BVp: Peel samples; INITIAL: Cupric reducing antioxidant capacity of the sample before digestion; PG: Cupric reducing antioxidant capacity antioxidant capacity of the sample after digestion in stomach; IN: Cupric reducing antioxidant capacity of the sample after digestion in small intestines. OUT: Cupric reducing antioxidant capacity of the sample that can not be recovered by GI tract. All values are given as mean values of triplicate runs.

Wootton-Beard et al. (2011) examined the total antioxidant capacity (TAC) and total polyphenol content (TPC) of 23 commercially available vegetable juices including red beet juice before and after in vitro digestion. They used several methods which were FRAP, DPPH, ABTS and Folin–Ciocalteu methods and the TAC of the studied vegetable juices founded to have a good stability through in vitro digestion, although there was a large variation in the responses of individual juices to this procedure. The enhancement could be attributed to the contribution of structurally transformed molecules and other antioxidant metabolites to total antioxidant capacity. The authors concluded that a number of methods underestimate the antioxidant capacity due to a failure to measure these. Hence, it is important to use multiple methods of analysis since there is no any single accepted assay for the measurement of TAC.

3.5.3 Bioaccessibility of Phenolics After GI Digestion by UFLC-MS/MS

The antioxidant activity of phenolic compounds is related to the composition as well as the concentration. Because not all polyphenols are absorbed with equal efficiacy. The bioaccessibility can be defined as the amount of compound solubilised in the small intestine and available for the subsequent absorption. This definition comprises the release of compounds from food matrices and their stability under the gastro-intestinal condition (Gawlik-Dziki, 2012; Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010).

There exist several parameters affecting the bioaccessibility of a compound including the initial concentration in the food matrix, the composition of the matrix and host-related factors, such as the physicochemical characteristics of the gastro-intestinal fluids and the presence of digestive enzymes. Mastication in the mouth initiates the digestive process breaking down the food matrix and causing the release of most of the phenolic compounds present in the food. Then, the food bolus is subjected to gastro-intestinal digestion where the presence of different hydrolytic enzymes and the physicochemical characteristics of the secretions (Tagliazucchi, Verzelloni, & Conte, 2012). It is known that the gastric acidity stabilizes the phenolic compounds and favour their release from the food matrix. On the contrary, several phenolic compounds (including phenolic acids) degraded at the mild alkaline pH of the small intestine (Helal et al., 2014; Tagliazucchi et al., 2010).

Additionally, the simulative in vitro digestion process might not have allowed sufficiently to show the microfloral activity of the small intestines in reality, therefore the interaction with the polyphenols could not be understood thoroughly. It has been reported that the effectiveness of polyphenol absorption in the digestive tract depends several factors (Manach et al., 2005). In order to evaluate deeply, factors having inverse and synergistic effects on the digestion mechanism of the human GI tract should be understood and the possible interactions of the multiple effect combinations in the in-vitro digestion should further be investigated.

UFLC/MS-MS analysis of phenolic acids of the flesh and peel extracts after in vitro GI digestion are represented in Table 3.14, Table 3.15 and Table 3.16

Table 3.14 : UFLC/MS-MS analysis of phenolic acid contents of IN fractions after in vitro GI digestion.

Phenolic acid	BVf			BVp		
	1.batch	2.batch	3.batch	1.batch	2.batch	3.batch
4 (p-)Hydroxybenzoic Acid	< 0	< 0	< 0	< 0	< 0	< 0
Vanilic Acid	8,57	1,1	6,95	1,57	< 0	6,24
Ferulic Acid	31,9	8,03	19,3	4,26	10,18	14,95
Syringic Acid	3,71	4,59	3,71	< 0	6,16	3,71
Chlorogenic Acid	4,13	4,11	4,14	4,09	4,13	4,12

BVf: Flesh samples. BVp: Peel samples. Results are expressed as ng per g fresh weight (n=3).

It is noticeable that 4-(p)hydroxybenzoic acid decreased significantly because of digestion process. It may be transformed into other structural forms. Results have shown that phenolic acids were degraded with different proportions during in vitro digestion. Lack of equal efficiacy exist in absorbance of polyphenolic compounds as it depends on various factors related with food matrix and gastro-intestinal media as well as the phenolic compound itself. Bermudez-Soto et al. (2007) suggested that dietary polyphenols are very sensitive to the mild alkaline conditions in the small intestine and that a good proportion of these compounds can be transformed into other unknown and/or undetected structural forms with different chemical properties and, consequently, different bioaccessibility, bioavailability and biological activity.

Table 3.15 : UFLC/MS-MS analysis of phenolic acid contents of PG fractions after in vitro GI digestion.

Phenolic acid	BVf			BVp		
	1.batch	2.batch	3.batch	1.batch	2.batch	3.batch
4 (p-)Hydroxybenzoic Acid	< 0	< 0	< 0	< 0	< 0	< 0
Vanilic Acid	11,15	11,36	9,97	5,3	7,64	7,63
Ferulic Acid	17,6	24,1	6,5	4,93	11,43	5,11
Syringic Acid	5,18	13,02	4,69	11,54	7,14	6,65
Chlorogenic Acid	4,15	4,13	4,13	4,12	4,21	4,58

BVf: Flesh samples. BVp: Peel samples. Results are expressed as ng per g fresh weight (n=3).

Table 3.16 : UFLC/MS-MS analysis of phenolic acid contents of OUT fractions after in vitro GI digestion.

Phenolic acid	BVf			BVp		
	1.batch	2.batch	3.batch	1.batch	2.batch	3.batch
4 (p-)Hydroxybenzoic Acid	< 0	< 0	< 0	< 0	< 0	< 0
Vanilic Acid	13,7	11,35	10,42	5,07	7,87	4,37
Ferulic Acid	10,42	22,5	7,1	4,38	4,26	10,17
Syringic Acid	7,14	13,5	1,76	3,71	6,16	2,26
Chlorogenic Acid	4,2	4,12	4,11	4,14	4,12	4,13

BVf: Flesh samples. BVp: Peel samples. Results are expressed as ng per g fresh weight (n=3).

4. CONCLUSIONS AND RECOMMENDATIONS

In this study, antioxidant capacity and bioaccessibility of phenolic acids were determined and compared after GI digestion using in vitro models. Thanks to this study, an underutilized local beetroot variety native to Turkey investigated for the first time including fleshes (BVf) and peels (BVp) and presented to food science and technology.

Betalain pigments, phenolic composition and contents were determined as well as total antioxidant capacity. Moreover, in vitro digestion was performed for phenolic compounds. Total betalain content was analyzed spectrophotometrically. Total phenolic content was determined by Folin-Ciocalteu method and total antioxidant capacity by DPPH and CUPRAC assays were performed before and after digestion. The experiments were carried out to three different biological replicates which were harvested at different times from various farms. The results of all assays indicated statistically significant differences between the batches and between peel and flesh samples. Peel samples showed higher values than flesh samples according to all assays performed.

In regards to the total antioxidant capacity methods, CUPRAC assay gave higher responses than the DPPH assay in accordance with the literature, although the antioxidant capacity of this beetroot variety was not as high as red beetroot. Total betalain content of this beetroot variety was also lower than the red beetroot. Total phenolic content was in a similar range with the reported values for red beetroot. However, the individual phenolic acid composition was seen different from the red beet and sugar beet. Similar chromatogram patterns of phenolic acids were observed for peel and flesh samples; contents differed depending on batches. The decreasing order of individual phenolic acids in peels was as follows: ferulic acid, chlorogenic acid, syringic acid, 4(*p-)*hydroxybenzoic acid, vanilic acid. On the other hand, the

decreasing order of individual phenolic acids in fleshes was as Ferulic acid, syringic acid, vanilic acid, 4(*p*-)hydroxybenzoic acid, chlorogenic acid.

Results from in vitro digestion demonstrated that differences in bioaccessibility of polyphenolics between the batches were significant and the reduction of TPC in peel samples is greater than in fleshes. In other words, the bioaccessibility of phenolics in fleshes is greater than the bioaccessibility of phenolics in fleshes. Additionally, phenolic acids were found to be stable in gastric digestion due to acidic conditions. However, their recoveries become lowered in IN fractions representing the compound passed into serum. The UFLC-MS/MS detection was done on phenolic acids from PG, IN and OUT fractions of GI digestion. Notably, no peak could be detected in IN, OUT and PG fractions for the 4(*p*-)hydroxybenzoic acid.

As the future study, further work is necessary to identify the bioaccessibility of betalain pigments in addition to the phenolic compounds as well as the betalainic profile because of the excessive health effects of these valuable phytochemicals. As mentioned in the literature review section, the positive health effects of foods are gaining importance today. Hippocrates said in 400 BC, “Let your food be your medicine; and your medicine be your food". Day by day, food industry seeks for new and bioactive compounds possessing health effects. With this respect, bioaccessibility studies are essential as the bioactive compounds are transformable in GI digestion. Although, the betalain content and antioxidant capacity of this beetroot variety is not as high as red beetroot, it is still valuable as the betalain pigment presents in a limited number of plant materials in nature. Moreover, the BC/BX ratioof betalainic plants are crucial as the ratio provides different shades in color. Being widely used in food industry, beetroots have a large and inexpensive potential to expand their utilization by local varieties. Conclusively, a multifunctional food additive is proposed.

REFERENCES

Apak, R., Guclu, K., Ozyurek, M., & Karademir, S. E. (2004). Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. Journal of Agricultural and *Food Chemistry*, 52, 7970-7981.

Apak, R., Güçlü, K., Özyürek, M., Karademir, S. E., & Altun, M. (2005). Total antioxidant capacity assay of human serum using copper (II)-neocuproine as chromogenic oxidant: the CUPRAC method. Free radical research, 39(9), 949-961.

Apak, R., Guclu, K., Ozyurek, M., Karademir, S.E., Erceg, E. (2006). The cupric ion reducing antioxidant capacity and polyphenolic content of some 44 herbal teas, *International Journal of Food Sciences and Nutrition*, 57: 292-304.

Apak, R., Güçlü, K., Demirata, B., Özyürek, M., Çelik, S.E., Bektaşoğlu, B., Berker, K.I., Özyurt, D. (2007). Review: Comparative Evaluation of Various Total Antioxidant Capacity Assays Applied to Phenolic Compounds with the CUPRAC Assay. Molecules, 12, 1496-1547.

Azeredo, H. M. C. (2009). Betalains: properties, sources, applications, and stability: A review. *International Journal of Food Science & Technology*, 44(12), 2365–2376.

Bach, V. (2012). *Sensory quality and chemical composition of culinary preparations of root crops* (Doctoral dissertation). Retrieved from http://dca.au.dk/

Bavec, M., Turinek, M., Grobelnik-Mlakar, S., Slatnar, A., Bavec, F. (2010). Influence of Industrial and Alternative Farming Systems on Contents of Sugars, Organic Acids, Total Phenolic Content, and the Antioxidant Activity of Red Beet (Beta vulgaris L. ssp. vulgaris Rote Kugel). J. Agric. Food Chem., 58, 11825–11831.

Ben Haj Koubaier, H., Snoussi, A., Essaidi, I., Chaabouni, M. M., Thonart, P., & Bouzouita, N. (2013). Betalain and Phenolic Compositions, Antioxidant Activity of Tunisian Red Beet (Beta vulgaris L. conditiva) Roots and Stems Extracts. International Journal of Food Properties, (just-accepted).

Bouayed, J., Deußer, H., Hoffmann, L., & Bohn, T. (2012). Bioaccessible and dialysable polyphenols in selected apple varieties following< i> in vitro</i> digestion vs. their native patterns. *Food Chemistry*, *131*(4), 1466-1472.

Brand-Williams, W., Cuvelier, M. E., Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensmittel-Wissenschaft und Technologie*, *28*, 25-30.

Breinholt, V., (1999). Desirable versus harmful levels of intake of flavonoids and phenolic acids. In: Kumpulainen, J., Salonen, J.E. (Eds.), Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease. The Royal Society of Chemistry, Cambridge, pp. 93–105.

Cai, Y.Z.; Sun, M.; Corke, H. (2003). Antioxidant activity of betalains from plants of the *Amaranthacae*. *Journal of Agriculture and Food Chemistry*, *51*, 2288–2294.

Canadanovic-Brunet, J.M., Savatovic, S.S., Cetkovic, G.S., Vulic, J.J., Djilas, S.M., Markov, S.L., Cvetkovic, D.D. (1998). Antioxidant and antimicrobial activities of beet root pomace extracts. Czech J. Food Sci., 29(6) 575–585.

Cao, G., Srfie, E. & Prior, R.L. (1996). Antioxidant capacity of tea and common vegetables. Journal of Agricultural Food Chemistry, 44, 3425-3431.

Castellanos-Santiago, E., & Yahia, E. M. (2008). Identification and quantification of betalains from the fruits of 10 Mexican prickly pear cultivars by high-performance liquid chromatography and electrospray ionization mass spectrometry. Journal of agricultural and food chemistry, 56(14), 5758-5764.

Czapski, J., Mikołajczyk, K., Kaczmarek, M. (2009). Relationship between antioxidant capacity of red beet juice and contents of its betalain pigments. *Pol. J. Food Nutr. Sci.*, 59 (2), 119-122.

Duthie, G. G., Duthie, S. J., & Kyle, J. A. M. (2000). Plant polyphenols in cancer and heart disease: Implications as nutritional antioxidants. Nutrition Research Reviews, 13, 79–106.

Felczyński K. & Elkner K. (2008). Effect of long-term or-ganic and mineral fertilization on the yield and quality of red beet (*Beta vulgaris* L.). *Veget. Crops Res. Bull*. 68: 111-125.

Gandía-Herrero, F., Escribano, J., García-Carmona, F. (2009). The role of phenolic hydroxy groups in the free radical scavenging activity of betalains. J Nat Prod, 72:1142–1146.

Gandia-Herrero, F., Jiménez-Atiénzar, M., Cabanes, J., García-Carmona, F., Escribano, J. (2010). Stabilization of the Bioactive Pigment of Opuntia Fruits through Maltodextrin Encapsulation. *J. Agric. Food Chem.*, 58 (19), 10646–10652.

Gandia-Herrero, F., Escribano, J., García-Carmona, F. (2012). Purification and Antiradical Properties of the Structural Unit of Betalains. *J. Nat. Prod.*, 75 (6), 1030–1036.

Gasztonyi,M.N., Daood,H., Takacs Hajos, M., Biacs, P. (2001). Comparison of red beet (Beta vulgaris var conditiva) varieties on the basis of their pigment components. *Journal of the Science of Food and Agriculture,*81 (9), 932-933.

Gawlik-Dziki, U. (2012). Changes in the antioxidant activities of vegetables as a consequence of interactions between active compounds. Journal of Functional Foods, 4, 872–882.

Gazzani, G., Papetti, A., Massolini, G. & Daglia, M. (1998). Antioxidative and pro-oxidant activity of water soluble components of some common diet vegetables and the effect of thermal treatment. Journal of Food Chemistry, 6, 4118-4122.

Gokhale, S.V., Lele, S.S. (2011). Dehydration of red beet root (Beta vulgaris) by hot air drying: process optimization and mathematical modeling. *Food Sci. Biotechnol.*, 20 (4) 955–964.

Georgé, S.; Brat, P.; Alter, P.; Amiot, M. J. (2005). Rapid determination of polyphenols and vitamin C in plant-derived products. J. Agric. Food Chem. 53, 1370–1373.

Georgiev, V. G., Weber, J., Kneschke, E., Denev, P. N., Bley, T., & Pavlov, A. I. (2010). Antioxidant activity and phenolic content of betalain extracts from intact plants and hairy root cultures of the beetroot Beta vulgaris cv. Detroit Dark Red. Plant Foods Human Nutrition, 65(2), 105–111.

Helal, A., Tagliazucchi, D., Verzelloni, E., & Conte, A. (2014). Bioaccessibility of polyphenols and cinnamaldehyde in cinnamon beverages subjected to< i> in vitro</i> gastro-pancreatic digestion. *Journal of Functional Foods, 7*, 506-516.

Huang, D., Ou, B., Prior, R.L. (2005). Review: The Chemistry behind Antioxidant Capacity Assays. J. Agric. Food Chem., 53, 1841−1856.

Imeh, U., & Khokhar, S. (2002). Distribution of conjugated and free phenols in fruits: Antioxidant activity and cultivar variations. Journal of Agricultural and Food Chemistry, 50(22), 6301–6306

Ismail, A., Marjan, Z. M., & Foong, C. W. (2004). Total antioxidant activity and phenolic content in selected vegetables. Food Chemistry, 87(4), 581-586.

Janiszewska, E. and Wlodarczyk, J. (2013). Influence of spray drying on beetroot pigments retention after microencapsulation process.*Acta Agrophysica, 20(2), 343-356.*

Kahkönen, M. P., Hopia, A. I., Vuorela, H. J., Rauha, J. P., Pihlaja, K., Kujala, T. S., & Heinonen, M. (1999). Antioxidant activity of plant extracts containing phenolic compounds. Journal Agricultural and Food Chemistry, 47, 3954–3962.

Kanner, J., Harel, S., & Granit, R. (2001). Betalains – a new class of dietary cationized antioxidants. Journal of Agricultural and Food Chemistry, 49(11), 5178–5185.

Kaur, C., & Kapoor, H. C. (2002). Antioxidant activity and total phenolic content of some Asian vegetables. International Journal of Food Science & Technology, 37(2), 153-161.

Kevers, C., Falkowski, M., Tabart, J., Defraigne, J. O., Dommes, J., & Pincemail, J. (2007). Evolution of antioxidant capacity during storage of selected fruits and vegetables. Journal of agricultural and food chemistry,55(21), 8596-8603.

Krajka-Kuzniak, V., Szaefer, V., Ignatowicz, E., Adamska, T., Baer-Dubowska, W. (2012). Beetroot juice protects against N-nitrosodiethylamine-induced liver injury in rats,
Food Chem. Toxicol., 50, 2027–2033.

Kujala, T.S., Loponen, J.M., Klika, K.D. & Pihlaja, K. (2000). Phenolics and betacyanins in red beetroot (Beta vulgaris) root: distribution and effect of cold storage on the content of total phenolics and three individual compounds. Journal of Agricultural and Food Chemistry, 48, 5338–5342.

Kujala, T.S., Loponen, J.M., Pihlaja, K. (2001). Betalains and phenolics in red beetroot (Beta vulgaris) peel extracts: extraction and characterisation. *Zeitschrift fur Naturforschung*, 56c, 343-348.

Kugler, F., Stintzing, F.C., Carle, R. (2004). Identification of Betalains from Petioles of Differently Colored Swiss Chard (Beta vulgaris L. ssp. cicla [L.] Alef. Cv. Bright Lights) by High-Performance Liquid Chromatography−Electrospray Ionization Mass Spectrometry. *Journal of Agricultural and Food Chemistry, 52* (10), 2975-2981.

Lee, E.J., An, D., Nguyen, C.T.T., Patil, B.S., Kim, J., Yoo, K.S. (2014). Betalain and Betaine Composition of Greenhouse- or Field-Produced Beetroot (Beta vulgaris L.) and Inhibition of HepG2 Cell Proliferation. *J. Agric. Food Chem.*, 62 (6), 1324–1331.

Liang, L., Wu, X., Zhao, T., Zhao, J., Li, F., Zou, Y., ... & Yang, L. (2012). < i> In vitro</i> bioaccessibility and antioxidant activity of anthocyanins from mulberry (< i> Morus atropurpurea</i> Roxb.) following simulated gastro-intestinal digestion. *Food Research International*, *46*(1), 76-82.

Manach, C., Williamson, G., Morand, C., Scalbert, A., & Remesy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. American Journal of Clinical Nutrition, 81, 230S–242S.

Mariko, N., Hassimotto, A., Genovese, M. I., & Lajola, F. M. (2005). Antioxidant activity of dietary fruits, vegetables and commercial frozen fruit pulps. Journal of Agricultural and Food Chemistry, 53, 2928–2935.

McDougall, G. J., Dobson, P., Smith, P., Blake, A., Stewart, D. (2005). Assesing potential bioavailability of raspberry anthocyanins using an in vitro digestion system. *Journal of Agricultural and food Chemistry*. 53, 5896-5904.

Mohdaly, A. A., Sarhan, M. A., Smetanska, I., & Mahmoud, A. (2010). Antioxidant properties of various solvent extracts of potato peel, sugar beet pulp and sesame cake. Journal of the Science of Food and Agriculture, 90(2), 218-226.

Morales-Soto, A., García-Salas, P., Rodríguez-Pérez, C., Jiménez-Sánchez, C., Cádiz-Gurrea, M. D. L. L., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2014). Antioxidant capacity of 44 cultivars of fruits and vegetables grown in Andalusia (Spain). Food Research International, 58, 35-46.

Nemzer, B., Pietrzkowski, Z., Spórna, A., Stalica, P., Thresher, W., Michałowski, T., & Wybraniec, S. (2011). Betalainic and nutritional profiles of pigment-enriched red beet root (Beta vulgaris L.) dried extracts. Food chemistry, 127(1), 42-53.

Ng, A.; Harvey, A. J.; Parker, M. L.; Smith, A. C.; Waldron, K. W. (1998). Effect of oxidative coupling on the thermal stability of texture and cell wall chemistry of beet root (*Beta vulgaris*). *J. Agric. Food Chem., 46*, 3365-3370.

Obata, Y., Senba, Y. & Koshika, M. (1963). Detection of Phenolic Compounds by Chromatography in Beet Sugar Molasses, Agricultural and Biological Chemistry, 27:5, 340-341.

Osorio-Esquivel, O., Álvarez, V. B., Dorantes-Álvarez, L., & Giusti, M. M. (2011). Phenolics, betacyanins and antioxidant activity in< i> Opuntia joconostle</i> fruits. Food Research International, 44(7), 2160-2168.

Ou, B., Huang, D., Hampsch-Woodill, M., Flanagan, J. A., & Deemer, E. K. (2002). Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. Journal of agricultural and food chemistry, 50(11), 3122-3128.

Palafox-Carlos, H., Ayala-Zavala, J. F., & Ganzalez-Aguilar, J. A. (2011). The role of dietary fiber in the bioaccessibility and bioavailability of fruit and vegetable antioxidants. Journal of Food Science, 76, R6–R15.

Pedreno, M. A., & Escribano, J. (2000). Studying the oxidation and the antiradical activity of betalain from beetroot. Journal of Biological Education, 35, 49–51.

Prior, R.L., Wu, X., Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 53(8):3101-3113.

Raven, P. H., Evert, R. F. & Eichorn, S. E. (2005). *Biology of plants*. New York, USA: W.H. Freeman and Company Publishers.

Ravichandran, K., Abdelrahman, R.A., Knorr, D., Smetanska, I. (2012). The effect of different processing methods on phenolic acid content and antioxidant activity of red beet. *Food Research International*, 48, 16–20.

Ravichandran, K., Thaw Saw, N.M.M., Mohdaly, A.A.A., Gabr, A.M.M., Kastell, A., Riedel, H., Cai, Z., Knorr, D., Smetanska, I. (2013). Impact of processing of red beet on betalain content and antioxidant activity. *Food Res. Int.*, 50, 670–675.

Robards, K., Prenzler, P. D., Tucker, G., Swatsitang, P., & Glover, W. (1999). Phenolic compounds and their role in oxidative processes in fruits. Food Chemistry, 66, 401–436.

Robbins, R. J. (2003). Phenolic Acids in Foods: An Overview of Analytical Methodology. *J. Agric. Food Chem., 51(10),* 2866- 2887.

Rubatzky, V. E., Quiros, C. F. & Simon, P. W. (1999). *Carrots and related vegetable umbelliferae*. New York, USA: CABI publishing.

Serris, G.S., Biliaderis, C.G. (2001). Degradation kinetics of beetroot pigment encapsulated in polymeric matrices, *J. Sci. Food Agric*. 81, 691–700.

Sing, R. P., Chidambara Murthy, K. N., & Jayaprakash, G. K. (2002). Studies on the antioxidant activity of pomegranate peel and seed extracts using in vitro models. Journal of Agricultural and Food Chemistry, 50, 81–86.

Singleton, V. L., & Rossi, J. L. (1965). Colorimetry of total phenolics with phosphomolybdicphosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144–158.

Sreeramulu, D. and Raghunath, M. (2010). Antioxidant activity and phenolic content of roots, tubers and vegetables commonly consumed in India. Food Research International, 43, 1017–1020.

Stintzing, F.C., Schieber, A., Carle, R. (2002). Betacyanins in fruits from red-purple pitaya, *Hylocereus polyrhizus*(Weber) Britton & Rose. *Food Chemistry*, *77*(1), 101–106.

Stintzing, F. C., Schieber, A., & Carle, R. (2003). Evaluation of colour properties and chemical quality parameters of cactus juices. *European Food Research and Technology*, 216, 303–311.

Stintzing, F. C., & Carle, R. (2004). Functional properties of anthocyanins and betalains in plants, food and in human nutrition. *Trends in Food Science and Technolgy*, 15, 19–38.

Sulaiman, S. F., Sajak, A. A. B., Ooi, K. L., & Seow, E. M. (2011). Effect of solvents in extracting polyphenols and antioxidants of selected raw vegetables.Journal of Food Composition and analysis, 24(4), 506-515.

Svenson, J. Smallfield, B.M., Joyce, N.I., Sansom, C.E., Perry, N.B. (2008). Betalains in Red and Yellow Varieties of the Andean Tuber Crop Ulluco (Ullucus tuberosus). *Journal of Agricultural and Food Chemistry*, *56* (17), 7730-7737.

Szopinska, A.A. & Gaweda, M. (2013). Comparison of yield and quality of red beetroots cultivated using conventional, integrated and organic method. *Journal of Horticultural Research*, 21(1): 107-114.

Tagliazucchi, D., Verzelloni, E., Bertolini, D., & Conte, A. (2010). In vitro bioaccessibility and antioxidant activity of grape berries polyphenols. Food Chemistry, 120, 599–606.

Tagliazucchi, D., Verzelloni, E., & Conte, A. (2012). The first tract of alimentary canal as an extractor. phytochemicals from solid food matrices during simulated digestion. Journal of Food Biochemistry, 36, 555–568.

Tavares, L., Figueira, I., Macedo, D., McDougall, G. J., Leitão, M. C., Vieira, H. L. & Santos, C. N. (2012). Neuroprotective effect of blackberry (< i> Rubus</i> sp.) polyphenols is potentiated after simulated gastrointestinal digestion. *Food Chemistry*, *131*(4), 1443-1452.

Tesoriere, L., Allegra,M., Butera,D., Livrea, M.A. (2004). Absorption, excretion, and distribution of dietary antioxidant betalains in LDLs: potential health effects of betalains in humans. *Am J Clin Nutr*, 80:941–5.

Tesoriere, L., Fazzari, M., Angileri, F., Gentile, C., Livrea, M.A. (2008). In Vitro Digestion of Betalainic Foods. Stability and Bioaccessibility of Betaxanthins and Betacyanins and Antioxidative Potential of Food Digesta. J. Agric. Food Chem,. 56, 10487–10492.

Toydemir, G., Capanoglu, E., Kamiloglu, S., Boyacioglu, D., de Vos, R. C. H., Hall, R. D., & Beekwilder, J. (2013). Changes in sour cherry (Prunus cerasus L.) antioxidants during nectar processing and in vitro gastrointestinal digestion. Journal of Functional Foods, 5, 1402–1413.

Velioglu, Y. S., Mazza, G., Gao, L., & Oomah, B. D. (1998). Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. Journal of Agricultural and Food Chemistry, 46, 4113–4117.

Vinson, J. A., Hao, Y., Su, X., & Zubik, L. (1998). Phenol antioxidant quantity and quality in foods: vegetables. Journal of Agricultural and Food Chemistry, 46, 3630–3634.

Vulic, J., Canadanovic-Brunet, J., Cetkovic, G., Tumbas, V., Djilas, S., Cetojevic-Simin, D., Canadanovic, V. (2012). Antioxidant and cell growth activities of beet root pomace extracts. *J. Funct. Foods.,* 4, 670–678.

Vulić, J. J., Ćebović, T. N., Čanadanović-Brunet, J. M., Ćetković, G. S., Čanadanović, V. M., Djilas, S. M., & Šaponjac, V. T. T. (2014). In vivo and in vitro antioxidant effects of beetroot pomace extracts. Journal of Functional Foods, 6, 168-175.

Wang, M., Li, J., Rangarajan, M., Shao, Y., La Voie, E.J., Huang, T. (1998). Antioxidative phenolic compounds from sage (Salvia officinalis). J Agric Food Chem. 46:4869–4873.

Wende, G.; Waldron, K. W.; Smith, A. C.; Brett, C. T. (1999). Developmental changes in cell-wall ferulate and dehydrodiferulates in sugar beet. *Phytochemistry*, *52*, 819- 827.

Wootton-Beard, P. C., Moran, A., & Ryan, L. (2011). Stability of the total antioxidant capacity and total polyphenol content of 23 commercially available vegetable juices before and after< i> in vitro</i> digestion measured by FRAP, DPPH, ABTS and Folin–Ciocalteu methods. *Food Research International*,*44*(1), 217-224.

Yu, L., Haley, S., Perret, J., Harris, M., Wilson, J. and Qian, M. (2002).Free radical scavenging properties of wheat extracts. J Agric Food Chem. 50:1619–1624.

APPENDICES

APPENDIX A: Calibration Curves

APPENDIX B: Anova Tables

APPENDIX A

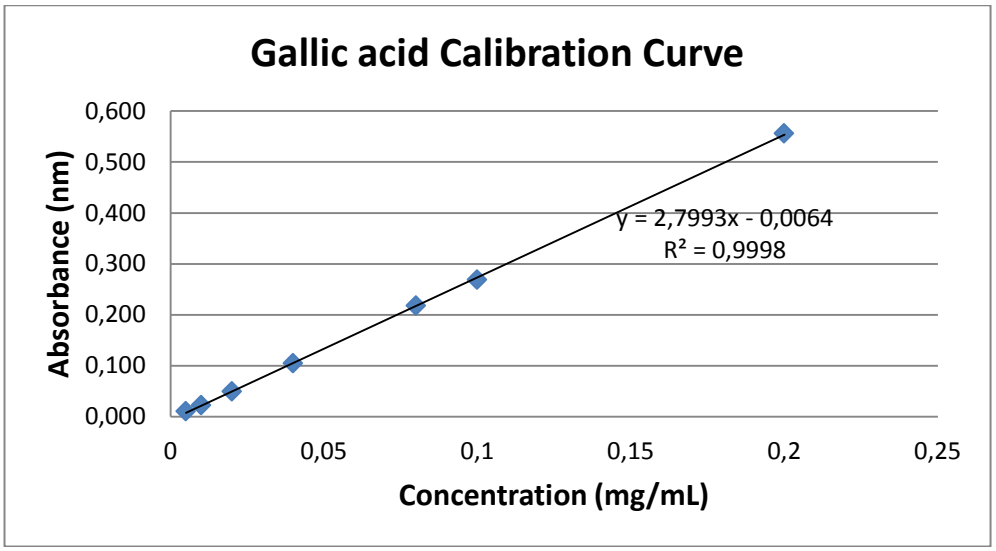


Figure A.1: Calibration curve of Gallic acid standard for Folin-Ciocalteu assay in 50% ethanol.

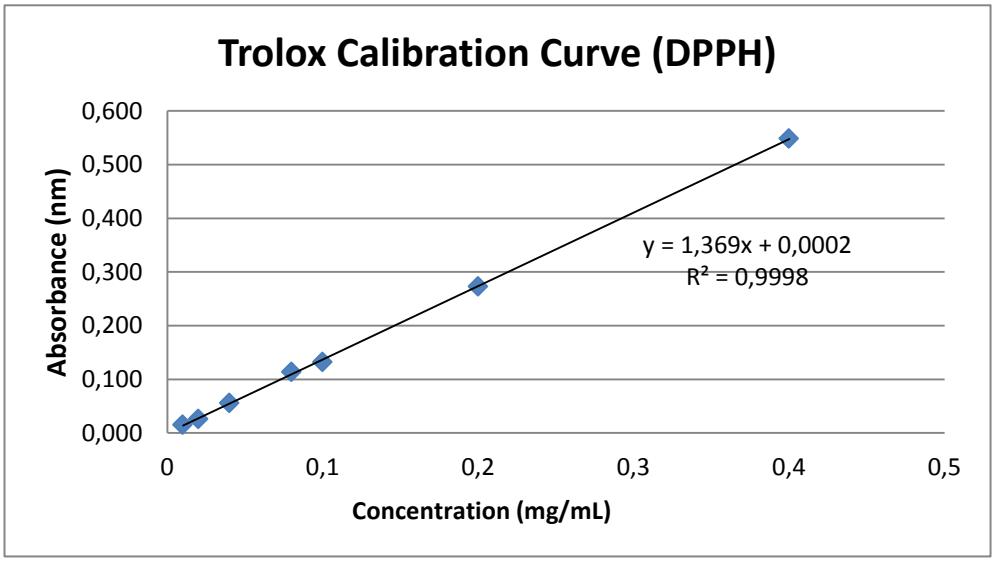


Figure A.2: Calibration curve of Trolox standard for DPPH assay in 50% ethanol.

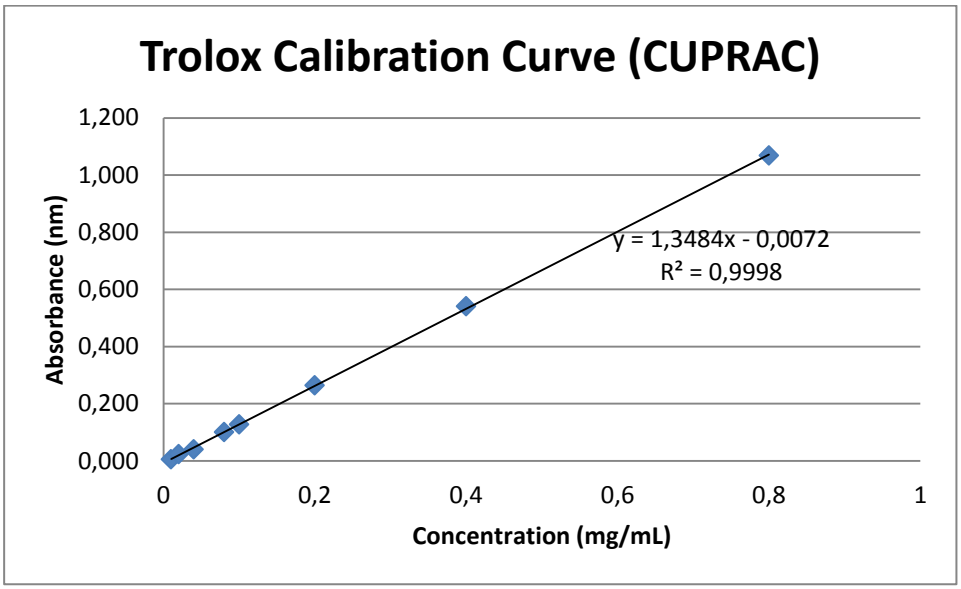


Figure A.3: Calibration curve of Trolox standard for CUPRAC assay in 50% ethanol.

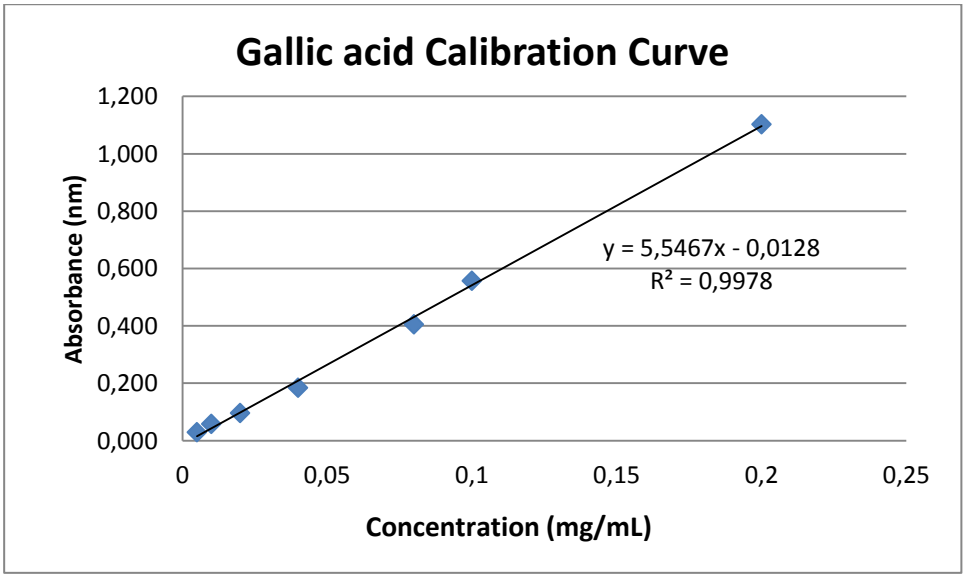


Figure A.4: Calibration curve of Gallic acid standard for Folin-Ciocalteu assay (IN, OUT, PG fractions).

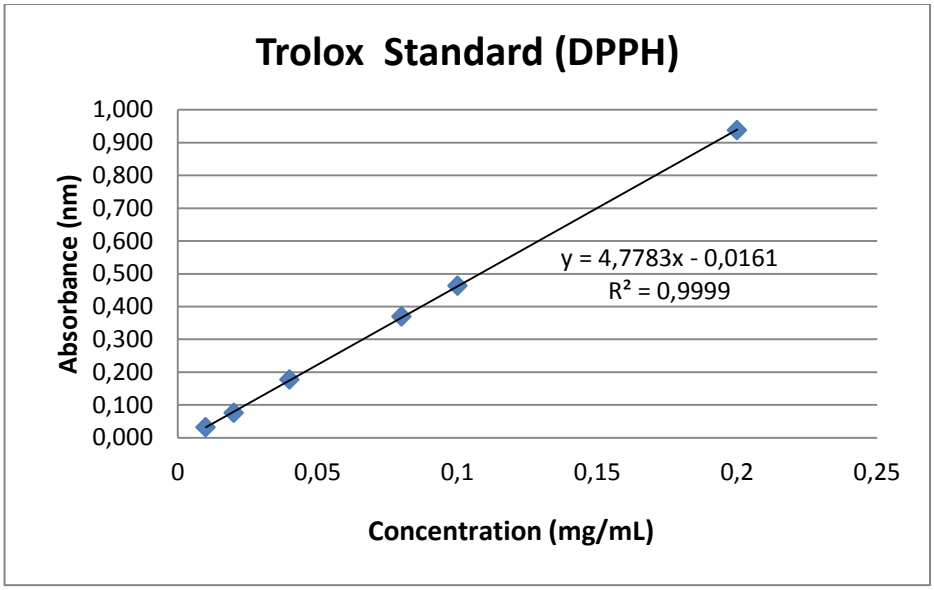


Figure A.5: Calibration curve of Trolox standard for DPPH assay (IN, OUT, PG fractions).

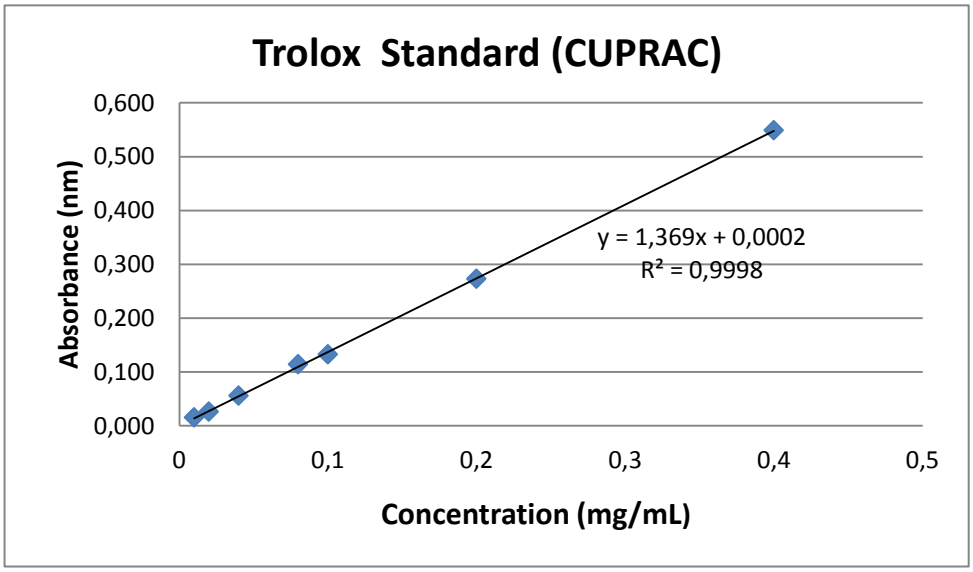


Figure A.6: Calibration curve of Trolox standard for CUPRAC assay (IN, OUT, PG fractions).

APPENDIX B

Table B.1 : Statistical analyses of initial samples.

Methods		Sum of Squares	df	Mean Square	F	Sig.
Folin-Ciocalteu	Between Groups	29,416	5	5,883	989,712	,000
	Within Groups	,071	12	,006		
	Total	29,488	17	1,006		
DPPH	Between Groups	5,031	5	,031	32,292	,000
	Within Groups	,374	12			
	Total	5,405	17			
CUPRAC	Between Groups	530,586	5	106,117	343,291	,000
	Within Groups	3,709	12	,309		
	Total	534,295	17			

Table B.2 : Statistical analyses of IN, OUT, and PG fractions for Folin-Ciocalteu assay.

Methods		Sum of Squares	df	Mean Square	F	Sig.
IN	Between Groups	313,320	2	156,660	42150,716	,000
	Within Groups	,185	4	,046	12,425	,000
	Total	836,982	36			
OUT	Between Groups	278,140	2	139,070	10886,112	,000
	Within Groups	,619	4	,155	12,122	,000
	Total	850,889	36			
PG	Between Groups	220,715	2	110,357	35663,049	,000
	Within Groups	,990	4	,247	79,979	,000
	Total	883,412	36			

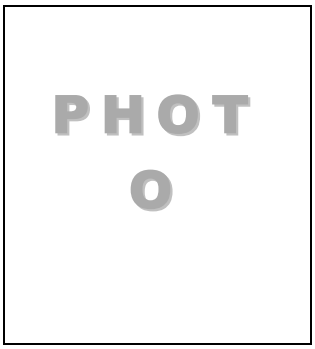
Table B.3 : Statistical analyses of IN, OUT, and PG fractions for DPPH assay.

Methods		Sum of Squares	df	Mean Square	F	Sig.
IN	Between Groups	Nd.	Nd.	Nd.	Nd.	
	Within Groups	Nd.	Nd.	Nd.	Nd.	
	Total	Nd.	Nd.	Nd.	Nd.	
OUT	Between Groups	Nd.	Nd.	Nd.	Nd.	
	Within Groups	Nd.	Nd.	Nd.	Nd.	
	Total	Nd.	Nd.	Nd.	Nd.	
PG	Between Groups	1,019	2	,510	32,262	,000
	Within Groups	,494	4	,124	7,824	,000
	Total	37,672	36			

Table B.4 : Statistical analyses of IN, OUT, and PG fractions for CUPRAC assay.

Methods		Sum of Squares	df	Mean Square	F	Sig.
IN	Between Groups	1724,496	2	862,248	5470,441	,000
	Within Groups	10,381	4	2,595	16,466	,000
	Total	3912,591	36			
OUT	Between Groups	1361,699	2	680,849	4356,895	,000
	Within Groups	14,197	4	3,549	22,713	,000
	Total	4027,322	36			
PG	Between Groups	1530,002	2	765,001	4929,836	,000
	Within Groups	3,964	4	,991	6,387	,000
	Total	3968,194	36			

CURRICULUM VITAE



Name Surname: Kadriye Nur KASAPOĞLU

Place and Date of Birth: ISTANBUL, 14.05.1988

E-Mail: kn.kspglu@gmail.com

EDUCATION:

B.Sc.: Integrated B.S. And M.S. Program in Teaching Chemistry, Boğaziçi University

PROFESSIONAL EXPERIENCE AND REWARDS:

- **The highest ranking graduate**

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS :

- Toktaş, B., Ecemiş, G., **Kasapoğlu, K.N.,** Özçelik, B. 2014. Changes in antioxidant capacity of black carrot during production of traditional fermented Shalgam beverage. *7th International Conference and Exhibition on Nutraceuticals and Functional Foods*, October 2014, Istanbul, Turkey.

- Altuntaş , Ü., Başaran, S.H., Yüzarı, S., **Kasapoğlu, K.N.,** Özçelik, B. 2014. Measurement of the Turkish consumers' level of awareness related to the safety of food packaging materials